COMPOSITION AND REPELLENCY OF ESSENTIAL OILS OF *Tagetes minuta* FROM DIFFERENT ZONES IN KENYA AGAINST BROWN EAR TICK (*Rhipicephalus appendiculatus*)

By

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DECLARATION

I hereby declare that this thesis is my original work and has not been presented for the award of any degree in any other university.

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DEDICATION

This work is dedicated to my aunt Turusira Nyaburi, my grandparents Charles Makang'a and Grace Nyambori and my mother Loice Kerubo not because they will ever understand any of this but because of their encouragement and material support they gave me for my entire educational achievement.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
DXP	1-Deoxyxyulose-5-phosphate
DXPS	1-Deoxy-D-xylulose-5-phosphate synthase
ECF	East Coast Fever
FAO	Food and Agriculture Organization
ICIPE	International Centre for Insect Physiology and Ecology
IIRR	International Institute of Rural Reconstruction
ITDG	Intermediate Technology Development Group
IUPAC	International Union of Pure and Applied Chemistry
GA	Guaiacol and allo-ocimene blend
GC	Gas chromatography
GC-MS	Gas chromatography linked to mass spectrometer
MEP	2-c-Methyl-d-erythritol-4-phosphate
NIST	National Institute of Standards and Technology
OA	β-Ocimene and allo-ocimene blend
OG	β-Ocimene and guaiacol blend
OGA	β-Ocimene, guaiacol and allo-ocimene blend
RD_{50}	Dose response at 50% confidence level
RD_{75}	Dose response at 75% confidence level
RT	Retention time
±SE	Standard error
SNK	Student Newman Keuls
VIE	Vance Integral Edition

ABSTRACT

Ticks are haematophagous ectoparasites capable of transmitting diseases to vertebrates and, therefore, constitute a threat to human, livestock and wildlife health. Though synthetic chemical acaricides have made a tremendous impact over the years in the control and management of the vector on livestock, ticks have developed resistance to most of them. In addition, the chemicals are toxic to non-target organisms. In some parts of Kenya, powdered parts of some plants, including those of *Tagetes minuta* L., are used to control ticks from the animals. The study aimed at characterizing and evaluating the repellency of the essential oils of *T. minuta* obtained from three agro-ecological zones (Nairobi-Kasarani, Western-Bungoma and Nyanza-Bondo) against *R. appendiculatus* Neumann 1901. The essential oils were isolated by hydrodistillation and analyzed by gas chromatography-linked mass spectrometry (GC-MS). The constituents were identified by comparing their mass spectra with those in the National Institute of Standards and Technology (NIST) libraries cinfirmed by co-injections wth standards. The repellency of the essential oils, selected constituents and blends was evaluated using tick climbing assay. The yields of the essential oils varied in the three agro-ecological zones: Nairobi-Kasarani 0.045±0.005 % w/w, Western-Bungoma, 0.039±0.005 % w/w and Nyanza-Bondo, 0.035±0.005 % w/w. That from Nairobi-Kasarani showed the highest repellency (70.06±2.76), followed by that from western-Bungoma (60.57±2.74) and Nyanza-Bondo (53.26±3.81). The compounds characterized from the oils were mainly monoterpenoids with some sesquiterpenoids. The major constituents were: β-ocimene (13.17 %), guaiacol (13.13 %), allo-ocimene (10.15 %), *trans*-tagetone (9.39 %), dihydrotagetone (8.20 %) and limonene (6.25 %) in Nairobi-Kasarani oil;, 2-pentanone (25.28 %), allo-ocimene (13.89 %), dihydrotagetone (8.73 %), β-ocimenone (5.24 %) and *trans*-tagetone (4.59 %) in Western-Bungoma oil; and, dihydrotagetone (9.46 %), 8,9-dehydrocycloisolongifolene (6.31 %), limonene (4.77 %), caryophyllene oxide (4.21 %) and *trans*-3,5-dimethyl-1,6-octadiene (2.66 %) in Nyanza-Bondo oil. Of the individual constituents and blends assayed, guaiacol and a blend of guaiacol with alloocimine were most repellent ($p \leq 0.05$, SNK). These results confirm the scientific basis of the traditional use of *T. minuta* to control the ticks and lay down the groundwork for more comphensive study of local plants for the development of eco-friendly and affordable tools for managing these ectoparasites.

CHAPTER 1

INTRODUCTION

1.1 Background

Ticks are obligate blood-feeding ectoparasites of vertebrates such as mammals, birds, amphibians and reptiles. Ticks belong to the sub-class Acari and sub-order Ixodidae which comprises three families, Ixodidae (hard ticks), Argasidae (soft ticks) and Nutalliellide (Sonenshine, 1991). Ixodid ticks spend several days feeding on the host while argasids feed rapidly lasting less than an hour (Krantz, 1978). Ixodid have only one nymphal stage while argasid ticks have at least two nymphal stages (Sonenshine, 1991). Both are important vectors of disease causing agents to humans and animals throughout the world. Ticks transmit the widest variety of pathogens of any blood sucking arthropod including bacteria, rickettsiae, protozoa and viruses. It is for this reason that ticks are of profound veterinary and medical importance (Sonenshine, 1991). Some human diseases caused by tick-borne pathogens include Lyme disease, babesiosis, Rocky Mountain spotted fever, tularemia and tick-borne relapsing fever (Sonenshine, 1993).

In most countries in Africa, a large proportion of people depend on livestock as a major source of natural, financial, physical and social capital in different ways and to varying degrees in smallholder dairy, crop–livestock and livestock-dependent economies. Unfortunately, *Rhipicephalus appendiculatus* have adverse effect on livestock industry in several ways (Snelson, 1975). The East Coast Fever (ECF) disease, caused by *Theileria parva* (Theiler, 1904) and transmitted by *R. appendiculatus*, is one of the major

constraints to cattle production and the expansion of dairy industry. The ECF causes considerable socio-economic losses to the livestock industry in sub-Saharan Africa (Norval *et al*., 1992; Olwoch *et al*., 2008). Among the 40-70 African known tick species, *R. appendiculatus* is the most economically important tick (Norval *et al*., 1992a; Walker *et al*., 2003). This is because it is a highly efficient vector of *T. parva, T. bovis* and *T. lawrencei*, which cause ECF, corridor disease and rhodesian malignant theileriosis, respectively. *R. appendiculatus* also transmits both Nairobi and Kisenye sheep disease viruses. The Nairobi sheep disease virus causes severe hemorrhagic gastroenteritis and high mortality in sheep and goats (Bugyaki, 1955).

ECF has been reported to cause half a million deaths of cattle per year in East Africa (VIE, 2002). In Kenya, it has been estimated that 50-80 % of national cattle population estimated at 10 million animals are exposed to tick infestation. Of the 10 million animals exposed to ticks, 1 % die of ECF each year (Mbogo *et al*., 1995; VIE, 2002) due to high costs associated with preventing and controlling the disease (Norval *et al*., 1992a). Kivaria (2006) estimated that the total annual cost of tick borne diseases in Tanzania was US$ 364 million of which 68 % was attributed to ECF. In another study in Tanzania, Kivaria *et al*. (2007) have estimated that the total losses annually per cow based on 2001 prices due to ECF mortality, morbidity and control and prevention practices to be at US$ 205.40. Heavy infestation of *R. appendiculatus* on cattle may also result in damage to the attachment sites, a fatal toxemia, suppression of immunity and reduced productivity (Norval *et al*., 1988; Pegram *et al*., 1989a).

1.2 Importance of ticks

Ticks as vectors of livestock diseases may represent one of the most important threats to the livestock industry in the tropics. As a direct effect, attachment to the host causes irritation of the skin, with subsequent ulceration and sometimes secondary bacterial infections. In addition, tick wounds may become infested by screw-worms or other agents of myiasis, and are also associated with the spread of bovine dermatophilosis caused by *Dermatophilus congolensis* van Saceghem 1915 (Norval *et al*., 1988). Heavy infestations of Ixodid tick species of *Amblyomma hyalomma* Koch 1844 and *Rhipicephalus* can result in anemia, particularly in small animals, and restlessness caused by the presence of large numbers of ticks. These ticks can lead to significant loss of weight and cause direct injury to hides due to tick bites (de Castro, 1997).

Ticks cause toxicosis in calves and wildlife which is characterized by an acute ascending flaccid motor paralysis caused by the injection of a toxin by certain ticks while feeding (de Castro, 1997). Examples are paralysis caused by the feeding of *Dermacentor andersoni* Stiles 1908, sweating sickness caused by *Hyalomma truncatum* Koch 1844, Australian tick paralysis caused by *Ixodes holocylus* Neumann 1899 and tick toxicosis caused by *Rhipicephalus* species (Drummond, 1983). Although affected animals may die, the paralysis is relieved if the ticks are removed quickly. Most domestic animal species appear to be susceptible to tick paralysis. The most common tick toxicosis is probably sweating sickness caused by an epitheliotropic toxin produced by *H. truncatum* (Drummond, 1983). Tick paralysis is most common in late winter and spring when the

adult ticks are active, but it can occur at any time if the weather is warm and humid (Stewart and de Vos, 1984).

Ticks are carriers of pathogens, which they transmit to animals during sucking of blood and cause a large variety of diseases (FAO, 1998). The major diseases include babesiosis, anaplasmosis, theileriosis (including East Coast Fever), and heart-water. Other diseases of lesser importance also cause severe economic losses to the livestock industry (Bram, 1984; Drummond, 1983). The presence, dynamics and the parasite load in ticks exert a major influence on the kinetics of transmission of tick-borne parasitic diseases (Morel, 1980). Generally, ticks become infested with the causative organisms of diseases while feeding on infected animals. Then the organism may be transmitted from stage to stage in the tick (like *T. parva* transmitted by *R. appendiculatus*) or from the female tick through the egg to the larvae, leading to an increase of several thousand times in vector potential (like *Babesia equi* transmitted by *Anocentor nitens* Neumann 1897). When the next stage or generation subsequently feeds on another animal, the organism is transmitted to that animal if it is susceptible to the disease (Drummond, 1983).

Tick-borne diseases generally affect the blood and/or lymphatic system by lowering its immunity (FAO, 1998). Tick fever organisms, like *Anaplasma marginale*, are significant causes of cattle morbidity in Australia, USA, China and other countries (CRC-VT, 2001). Cattle tick, *Boophilus microplus* Carestrini 1887, economically impact cattle production by transmitting pathogens that cause babesiosis (*Babesia bovis* and *Babesia bigemina*) and anaplasmosis (*A. marginale*) (Peter *et al*., 2005). Protozoans such as *T. parva*, *T.*

parva lawrenci and T. parva bovis transmitted by *R. appendiculatus* are the causative agents of ECF, corridor and January (Zimbabwean) diseases, respectively, in cattle (Uilenberg *et al*., 1982; Perry *et al*., 1990).

1.3 East Coast Fever

1.3.1 The mammalian host

East Coast Fever (ECF) is a protozoan disease of cattle caused by *T. parva* a pathogen transmitted by *R. appendiculatus*. *T. parva* parasitizes bovine leucocytes and erythrocytes in succession. The first typical clinical sign of ECF in cattle appears 7 to 15 days after attachment of infected ticks. This is seen as swelling of lymph nodes, drastic milk reduction in dairy animals, difficult and fast breathing, rise in temperature above 39.5 °C and reduced feeding due to loss of appetite (Young and Groocock, 1998). Animals can recover naturally or after treatment with theilericides, thus developing a long-lasting immunity giving almost complete protection from the disease (Burridge *et al*., 1972). Unfortunately, recovered or immunized animals remain carriers of the infection for life and, therefore, serve as a source of infection to other ticks (Young and Groocock, 1998).

1.3.2 Occurrence of East Coast Fever

The occurrence of the ECF is largely associated with the distribution of the vector tick species (*R. appendiculatus*) normally restricted to central, southern and eastern Africa (Figure 1) where the cattle hosts *Bos taurus* and *Bos indicus* Linnaeus 1758, the tick and the parasite share the same geographical location (Norval *et al*., 1992). However, ECF does not occur throughout the range of the vector ticks' distribution (Norval *et al*.,

1992b). This is because the distribution of the vector ticks even in the countries where they are known to occur commonly is not continuous, being influenced mainly by climate, vegetation and host availability. For example, high temperatures (>33 °C) experienced in some ecologically marginal areas within the range of the ticks' distribution, does not allow the development of theileria in the vector ticks (Young and Leitch, 1981). Further, in areas of extreme climatic conditions, ECF may fail to establish itself due to low number of ticks for long periods (Speybroeck *et al*., 2002).

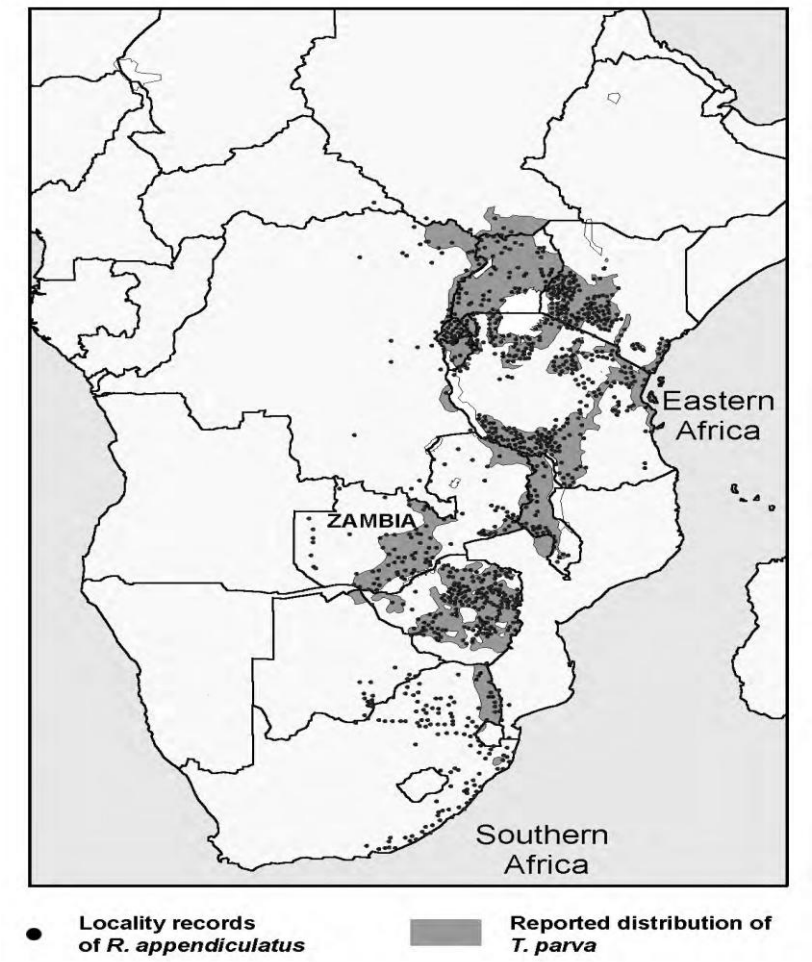


Figure 1: Distribution range of *T. parva* and *R. appendiculatus* (Chaka, 2001)

1.3.3 The epidemiology of East Coast Fever

The variables of ECF epidemiology can broadly be divided into pathogen and vector factors. The vector factors include: seasonality, abundance and the level of infection in the tick population (Norval *et al*., 1991). The level of infection in a tick population is influenced by ambient temperature, the behavior of the *T. parva*, the susceptibility of *R. appendiculatus* strains to *T. parva* infections, the presence of infected hosts and whether the infected hosts are clinical cases (animals which have not recovered from primary infections) or carriers (Young and Leitch, 1981).

Seasonality as a vector factor is attributed to when the weather conditions are unfavorable for the vectors to survive in all stages. Seasonal and climatic variations play an important role in epidemiology of *T. parva* infections due to the impact on vector density and activity. Seasonal variations typically correspond to vector breeding cycles and periods of peak population density (Seller and Maarouf, 1993; Gubler *et al*., 2001). Vectors may remain active and continue to transmit the pathogen, although the rate of transmission may be slowed by cold temperature, low vector abundance, frequency of blood feeding and rate of parasite maturation in a vector is diminished. During extended dry seasons transmission of the pathogen is completely terminated because the vector survival is minimal. Vector abundance depends on factors that affect breeding and survival. For example, climatic and environmental variations throughout the life cycle of a vector dramatically impact its abundance accounting for many variations in *T. parva* infections.

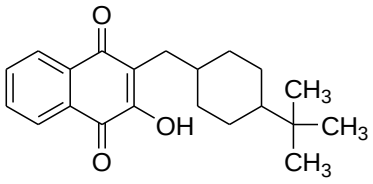
Carrier animals are those that have recovered from the primary infection but maintain piroplasms circulating in the blood at levels that are enough to infect ticks but not always detectable by routine diagnostic techniques (Medley *et al*., 1992). Ticks acquire higher infections when they feed on clinical cases than carrier cases. The latter present extremely low parasitaemia (Piroplams) hindering the development of high infections in ticks (Young *et al*., 1996). Carriers may include the African buffalo, which is the wildlife reservoir of infection (Burridge, 1975). Total level of infection in a population of ticks also depends on the numbers (abundance) of feeding stages on such clinical and/or carrier hosts. The abundance of feeding stages in an area is in turn influenced by host availability and macro- and micro-climatic requirements of the vector (Mulumba *et al*., 2000; Fandamu *et al*., 2005). The microclimate is modulated by vegetation cover (Minshull and Norval, 1982).

Climatic conditions vary throughout the range of *R. appendiculatus* life cycle. In response to climatic conditions, the ticks have evolved different behavioral and survival strategies. One such behavioral strategy is the diapause phenomenon exhibited by ticks from regions with a marked wet and dry season that enables ticks to delay feeding and hence oviposition so that the most vulnerable stages of their life cycles are synchronized with the incidence of favourable climatic conditions. Within the diapause phenomenon, ticks from different geographic areas have evolved different strategies for the initiation and termination of diapause (Berkvens *et al*., 1995; Madder *et al*., 2002). These behavioral strategies affect not only their abundance but also the number of generations per year. The number of generations per year among other factors has an effect on the

establishment of endemic stability (Billiouw *et al*., 2002; Marcotty *et al*., 2002), which is the epidemiological state of a population in which clinical disease is scarce despite high level of infection (Coleman *et al*., 2001).

1.3.4 The control of East Coast Fever

East Coast Fever can be controlled using various methods either singly or in combination. These methods are *T. parva* parasite control, immunization of susceptible hosts, and tick vector control using acaricides. *T. parva* parasite control aims at managing the parasite in the mammalian host which mostly takes the form of treatment using theilericidal chemicals such as buparvaquone (**1**), parvaquone (**2**) and halofuginone (**3**) (Dolan, 1999).



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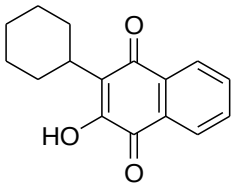

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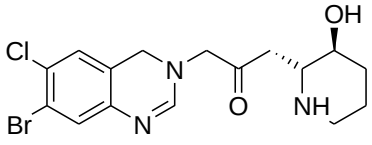

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For some years now, immunization has been and still is by an infection and treatment procedure (Marcotty *et al*., 2001; Mbao *et al*., 2006). This involves injecting cattle with live sporozoite material with the concomitant application of long-acting tetracyclines. Long acting tetracyclines slow down the division of schizonts and the schizont infected cells against which cellular immune responses are directed. Other vaccines tried include the 67kD circum-sporozoite antigen protein (p67) recombinant forms of which induce high antibody titres in cattle (Kaba *et al*., 2004; Musoke *et al*., 2005) and the

Polymorphic Immunodominant Molecule (PIM) (Toye *et al*., 1996). However, the p67 antigen only offers partial protection under field conditions (Kaba *et al*., 2004).

Vector control activities include practices such as movement restrictions which essentially entails keeping cattle away from infested pastures or herds, application of acaricides which take the form of plunge dips, sprays, pour-ons or hand-dressing preparations like tick grease and selection of tick resistant cattle. Choosing control options from the above in the context of integrated ECF control strategies depends on the production system, the prevalence of other tick-borne diseases and the epidemiological state of ECF for the area in question (Uilenberg, 1996; Billiouw, 2005). As already stated, the epidemiological state of an area is influenced by among other variables the vector factors. The vector factors of seasonality, abundance, vectorial competence and capacity are modulated by the environment and factor inherent to the vector itself. The latter may be genetic, phenotypic or a combination of both. Therefore an understanding of both genetic and phenotypic variation in the vector ticks infesting livestock in an area would contribute to the understanding of the ECF epidemiology of that area and subsequently aid in the choice and design of control strategies.

1.4 *Rhipicephalus appendiculatus*

1.4.1 Occurrence of *R. appendiculatus*

It is commonly known as Brown Ear Tick belongs to a genus of ticks in the family Ixodidae (hard ticks). Bovine cattle are the main host of *R. appendiculatus*, but buffaloes, elands and waterbucks serve as non-domestic hosts. Dogs, goats and sheep are also infested (Wanzala, 2009). *R. appendiculatus* is well adapted to domestic cattle and can be

maintained in all stages feeding on cattle but immature ticks can feed on the smaller hares and antelopes. On cattle, the immature stages of *R. appendiculatus* attach mainly on the neck and dewlap, eyelids, the cheeks, ears and muzzle. The adult *R. appendiculatus* prefer to feed on the ear pinna of bovids but not in the ear canal. In heavy infestations, adults can be found also around the eyelids, horns, upper neck even in the tail-brush and anus (Wanzala, 2009).

The occurrence and abundance of *R. appendiculatus* is affected by a number of factors, including the amount of vegetation cover (Norval, 1977), the abundance of suitable ruminant hosts (Norval and Lightfoot, 1982), climate (Norval *et al*., 1992) and acaricides used (Howell *et al*., 1981). The vegetation cover affects microclimate (Minshull and Norval, 1982), which is important for the survival of the free-living stages of tick (Branagan, 1973). Overgrazing and removal of trees reduce the vegetation cover and makes the *R. appendiculatus* to disappear (Norval *et al*., 1992). In the presence of hosts that have low level of resistance, the species becomes abundant (Lightfoot and Norval, 1982). Prolonged intensive acaricide treatment of livestock can cause local eradication of *R. appendiculatus* but the tick can spread again if control measures are stopped (Norval *et al*., 1992). *R. appendiculatus* prefers cool, shaded shrubby or woody savannas with at least 24 inches of annual rainfall. It is endemic from southern Sudan and eastern Zaire to eastern and South Africa and, can be found from sea level to 7400 feet (2300 meters) (Norval *et al*., 1992).

1.4.2 Life cycle of *R. appendiculatus*

The life cycle of *R. appendiculatus* (Figure 2) has three distinct life stages: larvae, nymph and adult. Larvae, nymphs and adults each go through a parasitic and free-living phase by a pattern of host seeking, feeding and off–the–host moulting. This developmental pattern is a typical three-host cycle where each unfed life stage (larvae, nymphs or adults) feeds on a separate host. Larvae which emerge from the egg have six legs. After obtaining a blood meal from a vertebrate host, they molt to the nymphal stage and acquire eight legs (Norval *et al*., 1992). Nymphs feed and molt to the next and final stage, the adult, which also has eight legs. After feeding once more, the adult female hard ticks lay one batch of thousands of eggs and then die. Only one blood meal is taken during each of the three life stages (Speybroeck, 2003). The time to completion of the entire life cycle may vary from less than a year in tropical regions to over three years in cold climates, where certain stages may become dormant until hosts are again available. Many hard ticks can go for several months without feeding until the environmental conditions become favorable (Wanzala, 2009).

After moulting, followed by a period of hardening and in certain instances dormancy, immatures and adults alike seek hosts, a process called questing by crawling up the stems of grass or perch on the edges of leaves on the ground in a typical posture with the front legs extended, especially in response to a host passing by (Sonenshine, 1993). Subsequently, these ticks climb on to a potential host which brushes against their extended front legs. Successful attachment on a suitable host at the predilection site is followed by feeding. Hard ticks feed for extended periods of time on their hosts varying

from several days to weeks depending on the life stage, host type, species of tick and environmental conditions (Branagan, 1974).

The outside surface of hard ticks actually grows to accommodate the large volume of blood ingested, which, in adult ticks, may be 200-600 times their unfed body weight (Sonenshine, 1991). After feeding for at least 4 days adult males and females mate on the host. Complete engorgement of females follows after mating. Fed females detach and seek a suitable microenvironment. Oviposition commences after a 3-10 day period of preovipositional development. Females lay a large number of eggs of approximately 4000 eggs. Period of oviposition may last up to a month depending on ambient temperatures. Males may remain on the host for 4-6 weeks and mate with successive batches of females (Branagan, 1974).

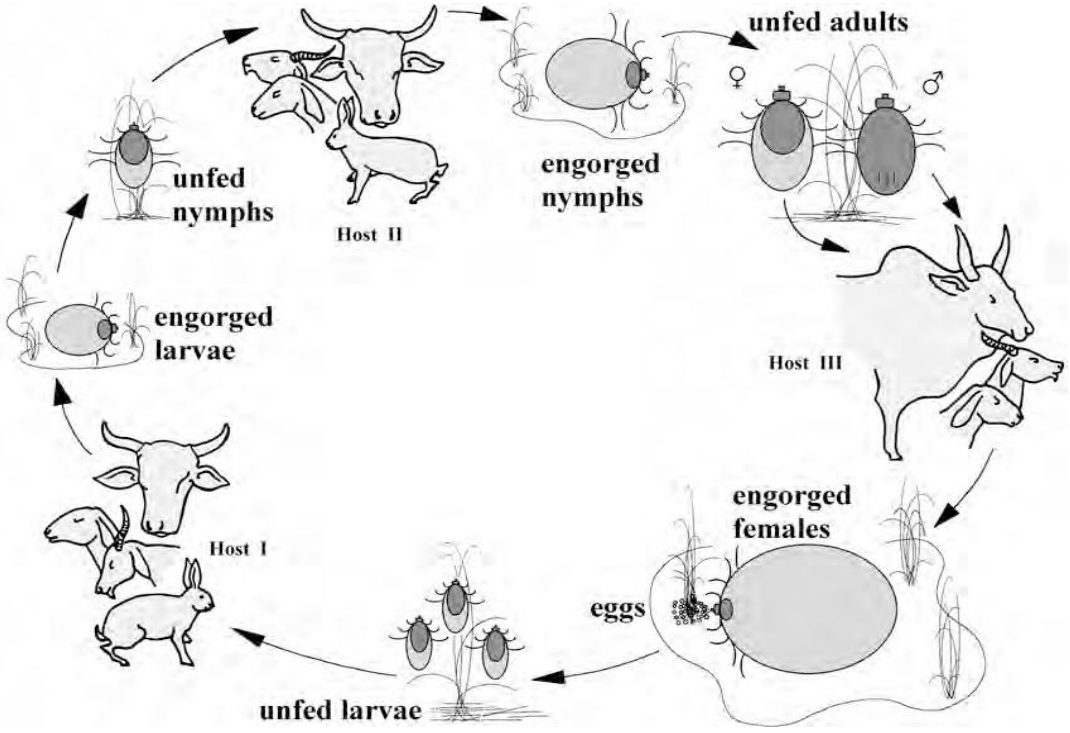


Figure 2: Life cycle of three-host tick, *R. appendiculatus* (Speybroeck, 2003)

1.4.3 Morphology of *R. appendiculatus*

R. appendiculatus was originally described by Neumann in 1901. Several redescriptions of the instars of *R. appendiculatus* have since been given (Walker *et al*., 2000). The adult *R. appendiculatus* has been described as a moderate-sized reddish-brown tick.

Figure 3: Male *R. appendiculatus*: dorsal view (Walker *et al*., 2000)

Male specimens (Figure 3) are characterized by a capitulum which is a lot longer than broad. The basis capitulum is variable; much broader than long in smaller males and only slightly broader in larger males, with short obtuse lateral angles at about the anterior quarter of its length. Palps are short and broad. Coxa I has a distinctly pointed strongly-sclerotized dorsal projection called the anterior process. A dorsal shield, the conscutum, extends from the tip of the scapular process to the distal end. The conscutum has scattered and moderate sized punctations. Cervical fields are broad and depressed with finely-reticulate surfaces. The marginal lines are distinct, extending anteriorly nearly to the eye level, delimiting one festoon posteriorly (Walker *et al*., 2003).

The posteromedian groove is long narrow and distinct, while the posterolateral grooves are short and broad. In smaller specimens the pattern of grooves and punctations may be

much reduced. In engorged specimens a slender caudal process is formed posteromedially. Eyes are marginal, almost flat and delimited dorsally by a very shallow groove. Ventrally spiracles are broadly comma shaped curving gently towards the dorsal surface. Adanal plates are large and well sclerotized tapering posterointernally to well-rounded points. Accessory adanal plates appear as small, short sclerotized points ((Walker *et al*., 2003)

Figure 4: Female *R. appendiculatus*: dorsal view (Sonenshine, 1993)

In contrast, the capitulum of females (Figure 4) is slightly longer than broad and the basis capitulum has broad lateral angles overlapping the scapulae. The porose areas on the basis capituli are round. Palps are short broad and bluntly rounded appically. The dorsal shield now called the scutum is longer than broad but may be approximately equal in length and width in smaller specimens. The eyes are located at the widest point of the scutum, are marginal, almost flat and are delimited dorsally by a faint groove. Cervical fields are broad and depressed. Ventrally the genital aperture is shaped like the tip of the tongue (Walker *et al*., 2000).

Nymphs have a capitulum that is wider than it is long (Figure 5). Their basis capitulum is approximately twice as broad as it is long. The lateral angles of the basis capitulum are in the anterior half of its length. Ventrally the basis capitulum has short blunt spurs on the posterior margin. Palps are short and broadly rounded apically. The scutum is wider than it is long with the posterior margin forming a broad smooth curve. Eyes are located at the widest point of the scutum, are mildly convex and are dorsally delimited by a shallow groove. The cervical fields are broad and slightly depressed and extend almost to the posterior margin of the scutum. Ventrally each coxa I has a long narrow external spur and a shorter broader internal spur; coxae II to IV each with a short sharp external spur only (Sonenshine, 1993)

Figure 5: *R. appendiculatus* nymph: dorsal view

The larval capitulum is broader than long (Figure 6). The width of the basis capitulum is a little over two times longer than the length with very short blunt lateral angles. Palps are constricted proximally and flattened apically. The scutum is much broader than long forming a smooth broad curve at the posterior margin. Eyes are at the widest point of the scutum, almost flat and delimited dorsally by a faint groove. Cervical grooves are short and slightly convergent. Ventrally coxa I each has a broad blunt spur; coxae II and III

each with a broad ridge-like spur. The above descriptions notwithstanding, it should be noted that like other rhipicephalids *R. appendiculatus* shows a wide range within-species variation in morphological appearance (Walker *et al*., 2000).

Factors affecting the wellbeing of immature stages cardinal of which is nutrition have a major effect on morphological variation within a species (Hoogstraal, 1956). Nutrition may be affected by crowding on the host, non availability of suitable hosts and degree of host resistance to tick feeding. The result is morphological variations of the shape of the basis capituli, punctation of the scutum (Walker *et al*., 2000) and robustness (Hoogstraal, 1956). Variation in size has been observed in geographically differentiated populations of *R. appendiculatus* (Speybroeck *et al*., 2004). Within the same geographical area specimens collected in the hotter and drier valley areas tend to be smaller (Chaka *et al*., 1999) and more punctate than those collected on the wetter and cooler plateau areas. In some areas with a more than one adult phenology per year there is variation in size of the different temporal adult groups (Chaka *et al*., 1999).

Figure 6: *R. appendiculatus* larva: dorsal view

1.5 Control methods of *R. appendiculatus*

1.5.1 Chemical control

The first application of ixodicides to control ticks on cattle was made by treating the infested cattle with various oils such as paraffin but without much success (Harrison *et al*., 1973). The main method has been the use of chemical acaricides. Acaricides are synthetic chemicals used to control and kill ticks on livestock or in the environment. They are applied in such a manner that the ticks are killed but will not harm the livestock. Chemical control is considered as one of the best methods, but it has certain implicit drawbacks such as the presence of residues in the milk and meat, acaricides transform into poisonous substances which bioaccumulate in the environment, and are toxic to both man and animals. The development of new acaricides is a long and expensive process (Graf *et al*., 2004)

Recently, it was shown that ticks have developed resistance against most of acaricides (Martins *et al*., 1995). This has led to the search for alternative and highly effective control approaches, to control tick infestations both on the host animal and the surrounding environment to prevent tick re-infestation. A wide range of acaricides, including arsenical, chlorinated hydrocarbons, organophosphates, carbamates and synthetic pyrethroids have been used for controlling ticks on livestock (Mitchell, 1996).

Use of arsenicals was the first effective method for controlling ticks and tick-borne diseases, and was used in many parts of the world before resistance to the chemical became a problem. The discovery and use of arsenical solutions in dipping vats for

treating cattle to protect them against ticks revolutionized tick and tick-borne disease control programmes. It was first used for tick control in 1893 in South Africa because the arsenic was inexpensive, stable and water soluble (Bekker, 1960). It was mostly used in the form of water soluble compounds like sodium arsenite. Usually, arsenic oxide (As_2O_3) has been used for many years in dipping vats to control ticks, especially ticks of the genus *Boophilus*. Arsenic dips were used successfully to eradicate *Boophilus* ticks from the southern United States. Unfortunately, arsenic has a very short residual effectiveness of less than one to two days, and in most areas of the world *Boophilus* ticks have become resistant to arsenic (Drummond, 1983).

Chlorinated hydrocarbons are synthetic acaricides that were developed when many species of ticks became resistant to arsenicals (Matthewson and Baker, 1975; Angus, 1996). They include dichlorodiphenyltrichloroethane (DDT), benzene hexachloride (BHC), chlordane, heptachlor, aldrin, dieldrin, methoxychlor and toxaphene (Graham and Hourrigan, 1977). Chlorinated hydrocarbon acaricides are very persistent and have been used extensively throughout the world for controlling ticks because of their high efficacy and good protection against tick re-infestation (Hitchcock and Mackerras 1947; Legg, 1947). Of particular interest are benzene hexachloride and toxaophene (Drummond, 1983). Their mode of action is by interfering with nerve conduction of ticks (Solomon, 1983). These compounds have been withdrawn from the market because of their high toxicity and long life span, and residues accumulated in treated food animal tissues particularly the fat led to development of a significant resistance (Spickett, 1998).

Organophosphates were introduced around 1950 as a replacement for the chlorinated hydrocarbons to which significant resistance had occurred (Shanahan and Hart, 1966). Organophosphates are esters of phosphoric acid which include diazinon, dioxathion, carbophenothion, coumaphos and ethion. They have a wide range of activities against ticks at relatively low concentrations. However, they have a shorter residual effectiveness than chlorinated hydrocarbons and a greater risk of causing acute toxicity in livestock (Drummond, 1983). Resistance in ticks was first recognized in 1963 and several tick species are now known to be resistant to organophosphorous acaricides (Wharton, 1967). Carbamates are esters of carbamic acid such as carbaryl and promacyl, and they closely resemble the organophosphates (Spickett, 1998). They are a little more toxic than the organophosphates for mammals, but are much more expensive.

1.5.2 Mechanical control

This is done by handpicking, whereby in certain communities, animals are held in a crutch facility and ticks are picked one by one and either burned or buried (Manna *et al*., 2001). This practice is also conducted during milking and cleaning of livestock sheds by women (Marina *et al*., 2001). Some ticks after being picked from respective host animals are given to chicken as food supplement. Handpicking was done as a communal cultural practice to reduce tick burden on heavily infested animals (Mathias-Mundy and Mc-Corkle, 1989). However, this method is tedious, time consuming and is not sustainable for a large herd of cattle because it involves much labor.

1.5.3 Biological control

This involves use of biocontrol agents, mainly natural enemies which include insectivorous birds, parasitoid wasps, nematodes, *Bacillus thuringiensis* bacteria, and deuteromycete fungi (largely *Metarhizium anisopliae* Sorok and *Beauvaria bassiana* Vullis) (Samish and Rehacek, 1999), which reduces the density of the target population or even eliminates it. Isolates of *M. anisopliae* and *B. bassiana* are pathogenic against ixodid tropical ticks *R. appendiculatus*, *Ambloymma variegatum* Fabriscius 1794, *Rhipicephalus (Boophilus) decoloratus* Koch 1844, *Rhipicephalus sanguineus* Latreille 1806, *Rhipicephalus (Boophilus) microplus* Canestrini 1888 and other hard ticks such as *Ambloymma americanum* Linnaeus 1758, and *Ambloymma maculatum* Koch 1844 in the laboratory (Kaaya *et al*., 1996; Frazzon *et al*., 2000; Benjamin *et al*., 2002; Kirkland *et al*., 2004a: 2004b).

In field experiments carried out by Kaaya (2002b) and Benjamin *et al*. (2002), aqueous suspensions of *M. anisopliae* sprayed on vegetation, reduced *R. appendiculatus* larvae and *Ixodes scapularis* Say 1821 unfed adults. Research at ICIPE identified a strain of *M. anisopliae*, ICIPE 7, as highly pathogenic against *A. variegatum* (Kaaya *et al*., 1996). The fungus was originally isolated from *A. variegatum*. Also, Maranga *et al*. (2006) developed a fungus-treated trap baited with semiochemicals. The trap was baited with semiochemicals that attracted *A. variegatum* to the fungus, became infected on contact and died.

Chickens (*Gallus gallus* Linnaeus 1758) confined with cattle in Africa have been reported to ingest an average of 338 ticks per bird during 5.5 hours and they seem to prefer *R. appendiculatus*, which concentrate close to the ears and eyes of cattle. Other experiments found that the birds ate from 10 to 81 ticks per bird per hour of foraging. At high tick concentrations, an average of 69 % of the ticks was consumed by chickens (Hassan *et al*., 1991: 1992; Dreyer *et al*., 1997). Thus, their consumption of ticks depends largely on alternative food availability and the density of the tick population. In literature, more than 257 tick biocontrol agents are mentioned, comprising 100 species of pathogens, seven parasitoids and 150 predators (Samish and Alekseev, 2001). During the past decades, interest in developing biological methods for tick control using birds (Couto, 1994), parasitoids (HU *et al*., 1998), entomo-pathogenic nematodes (Samish, 2000), entomo-pathogenic fungi, arthropods (Samish and Alekseev, 2001) and bacteria (Hassanain *et al*., 1997) has gained momentum worldwide because of limited impact of these organisms on the environment.

1.5.4 Host resistance

Cattle host resistance is an acquired characteristic that enables an animal to limit the establishment, growth rate and persistence of a parasite population (Coop and Kyriazakis, 1999). Each animal develops its own level of resistance in response to tick challenge; the level may be high (as in most indigenous zebu cattle) or low (as in most exotic European cattle), but a wide range of resistance occurs in all breeds of cattle not only in zebu×European breeds, but also within European breeds. However, selection for resistance for susceptibility must at present be based on tick numbers surviving on cattle

exposed either naturally or artificially to tick challenge. Host resistance expressed by an animal's ability to prevent the maturing of large numbers of ticks, and disease immunity, are survival mechanisms for the host and for external and internal parasites.

Different cattle breeds have diverse tick resistance ability due to host-specific physiological and immunological reactions reflected in their ability to reject tick attachment. For example, indigenous breeds the Zebu (*B. indicus*) and Sanga (*B. taurus and B. indicus* cross breed) cattle carry significantly fewer ixodid ticks than exotic European (*B. taurus*) breeds of cattle (Utech and Wharton, 1982). Consequently, tick infestation increases as the proportion of European genes in an animal increases (Lemos *et al*., 1985). Zebu breeds and their crosses are used as a means of controlling tick ectoparastic stages on livestock while retaining high productivity (Hayman, 1974; Mason, 1974; Turner, 1975).

The introduction of zebu cattle to Australia has positively revolutionalized the control of *B. microplus* on that continent as zebu breeds were successfully exploited in cattle programmes to develop tick resistant cattle breeds that limited the impact of *B. microplus* infestation (Seifert, 1984). Use of resistance cattle as a means of tick control is also becoming important in Africa, Asia and the America (Silva *et al*., 2007). Highly resistant cattle keep overall tick population very low as compared to those cattle with low resistance in the same herd that harbor more ticks in certain seasons (Solomon and Kaaya, 1996).

1.5.5 Cultural control measures

Cultural preventive management measures and practices employed in livestock tick control and management has long been used by various ethnic communities engaged in traditional animal husbandry. These measures aimed at reducing the risk of animals from being infested by ticks. They include burning of pasture land suspected to be infested with ticks in various stages of development directly kills ticks while ploughing grazing fields buries them and eventually they die (ITDG and IIRR, 1996), bush clearance to keep the tick population low by destroying their micro-habitats (particularly those close to homestead or frequently visited grazing grounds, Manna *et al*., 2001), hanging a bouquet of flowers/leaves at the doors, windows and in the roofs of cattle shade to repel ticks, and growing certain plant species with repellent properties around the boma (ITDG and IIRR, 1996). In addition, during migratory herding, areas infested with ticks are avoided at the times of the year when the population is at its largest (Sutherst, 1987; Sykes, 1987).

Herd distribution was practiced appropriately in which certain areas were used by cattle and small ruminants, or only by camels, depending on the varying spread of tick infestation specific to particular types of animals (Manna *et al*., 2001). Pasture spelling and rotation of pastures has been used in controlling one-host ixodid tick *B. microplus* on dairy farms in Australia (Sutherst *et al*., 1979; David, 2005).

Host removal and habitat interference can be directed against both the free-living and parastic stages of ticks (Aiello and Mays, 2003). The free living stages of most tick

species both argasid and ixodid, have specific requirements in terms of microclimate and are restricted to particular microhabitats within the ecosystems inhabited by the hosts. Destruction of these microhabitats reduces the abundance of ticks. Alteration of the environment by removal of certain types of vegetation has been used in the control of *A. americanum* in recreational areas in south eastern USA and in the control of *ixodes rubicundus* Neumann 1904 in South Africa. Also, removal of alternate hosts or hosts of particular stage of the life cycle can reduce significantly the abundance of tick species as this may starve ticks to death depending on the starvation period (Aiello and Mays, 2003). This approach can be used occasionally to control the three-host ixodid ticks such as *Ambyomma hebraeum* Koch 1844 and *R. appendiculatus*.

1.6 Application methods of acaricides

Various methods including dipping, spraying, handdressing, ear tagging or pour on have been used to apply acaricides to protect livestock against ticks. Direct application of acaricides to animals is the most popular method of controlling ticks on livestock (Drummond, 1983). Applications of acaricide to tick-infested cattle via dipping or spraying can be equally effective under ideal conditions with proper handling of equipments without injuring animals and subsequent dilution of a product (George, 2000).

Dipping is a method where animals are immersed in a dipping vat containing a solution of acaricides. This is the usual way of treating large numbers of animals. By 1893 in Australia, Africa and the United States, the use of dipping-vats to immerse tick-infested

cattle in a variety of chemical agents was a component of the effort to control the ticks and tick-borne diseases affecting cattle (Matthewson and Baker, 1975). A variety of tickicides including cottonseed oil, fish oil, crude petroleum, kerosene, tobacco extract, soap and a combination of sulphur and kerosene were among hundreds of possible acaricides tested for dipping (Mohler, 1906; Angus, 1996). In general, dipping vats provide a highly effective method of treating animals with acaricides for tick control. However, their immobility, high initial cost of construction and the cost of the acaricides may make vats impractical for many small scale operations. Also, dipping vats must be managed carefully so that the dips are maintained at the proper concentration and the cattle are dipped properly (Drummond, 1983).

Spraying involves application of fluid acaricides to an animal by means of spraying equipment. This method is suitable for small scale operators who cannot afford dipping vats. Spraying equipment is highly portable and only small amounts of acaricides need to be mixed for a single application (Barnett, 1961). However, spraying is generally less efficient in controlling ticks than immersion in a dipping vat because of problems associated with applying the acaricides thoroughly on all parts of the animal's body (Drummond, 1983).

Hand dressing is mainly used at the predilections sites which are not effectively treated by spray or dips for certain tick species on part of the body. The inner parts of the ear, under part of the tail, the tail brush and the areas between the teats and the legs in cattle with large udder, are especially liable to escape treatment. Acaricides may be applied to

these sites by hand is termed as hand dressing (Barnett, 1961). The application of insecticides with aerosols and in oils, smears and dusts by hand to limited body areas is time-consuming and laborious, but in certain instances it may be more effective and economical in terms of cost of acaricide than treating the entire animal (Drummond, 1983).

1.7 Problem statement and justification

Tick control and management heavily rely on application of synthetic chemical acaricides. This overdependence on acaricides has far-reaching implications such as bioaccumulation causing environmental pollution, development of resistant tick strains and elimination of ticks only from the host animal thus allowing re-infestation. Also, these synthetic acaricides are expensive to resource-limited farmers and toxic to both man and animals. This has made the current situation unsatisfactory and there is need to develop alternative and more effective control methods that are accessible to resource-limited farmers.

One approach to discovering alternative tick-control tools is to study scientifically the ethnobotanical practices. These practices have been used traditionally to repel and/or kill the ectoparasites. In a previous study, the essential oil of *T. minuta* collected from Bungoma in western Kenya was found to have varying repellent effect against *R. appendiculatus* (Wanzala, 2009). This essential oil was not studied in detail to identify specific constituents and/or blends responsible for repellency. Moreover, possible variation in the composition of different chemotypes of *T. minuta* growing in different

agro-ecological zones and, therefore, repellency of their essential oils against *R. appendiculatus* was not evaluated. Further downstream development and effective application of the plant in tick control depends upon comprehensive information relating to such variations.

1.8 Hypotheses

i. Essential oils of *T. minuta* growing in different agro-ecological zones contain varying levels of major constituents.

ii. Repellent property of the essential oils of *T. minuta* against *R. appendiculatus* is largely due to additive and/or synergistic effects of different constituents

iii. Essential oils of *T. minuta* located in different agro-ecological zones may repel *R. appendiculatus* differently due to different proportions of individual constituents.

1.9 Objectives

1.9.1 General objective

To investigate the repellency of the essential oils obtained from *T. minuta* growing in different agro-ecological zones against *R. appendiculatus* and to elucidate the relationship between their repellency and composition.

1.9.2 Specific objectives

i. To compare repellency of the essential oil of *T. minuta* located in three different agro-ecological zones in Kenya.

ii. To compare the relative amounts of the major chemical constituents of the essential oil of *T. minuta* obtained from the three zones.

iii. To identify chemical constituents and/or blends primarily responsible for repellency.

1.10 Limitation and scope of the study

Tagetes minuta species used was sampled from only three different agro-ecological zones: Nairobi-Kasarani, western-Bungoma and Nyanza-Bondo because of time limit. Only three available synthetic standards were used in follow up bioassays of synthetic blends against *R. appendiculatus*.

1.11 Background on agro-ecological zones from where *T. minuta* was sourced

An agro-ecological zone is a land resource mapping unit defined in terms of climate, landform and soils, and/or land cover, and having a specific range of potentials and constraints for land use (FAO, 1996).

Nairobi is classified under the medium potential zones in Kenya (Sombroek *et al*., 1982). The average annual rainfall in Nairobi is about 900 mm, but the actual amount in any one year may vary from less than 500 mm to more than 1500 mm. There are two rainy seasons, from mid-March to the end of May (long rains) and from mid-October to mid-December (short rains). The dates on which these rainy seasons start and end are very variable; in fact the beginning and end of a wet season are seldom, if ever, well defined (Sombroek *et al*., 1982).

Western is classified among the high potential zones in Kenya and well away from Nairobi. It is located in the west of the Eastern Rift Valley with an area of 8,361 km². The climate is mainly tropical with variations due to altitude. Kakamega district is mainly hot and wet most of the year while Bungoma district is colder but just as wet. Busia district is the warmest while the hilly Vihiga District is the coolest. The entire province experiences very heavy rainfall all year round with the long rains in the earlier months of the year. Subsistence and cash crop farming is the main economic activity in the province with sugar cane being the preferred medium to large scale crop. Vihiga district has large tea plantations and quarrying for construction materials is a significant activity in the hilly district. Dairy farming is also widely practiced in Vihiga. Western Kenya has many large factories including sugar processing plants (Mumias Sugar plant based at Mumias to the west of Kakamega) and Pan Paper Mills factory in Webuye.

Nyanza is among the high potential zones in Kenya together with western and is located 385 kilometres from Nairobi. It has a total area of about 32912 km^2 out of which 15979 km^2 is under water. Generally, Nyanza Province is characterized by sufficient rainfall for agricultural production. The rainfall pattern in the province is unevenly distributed throughout the year where highland regions of Kisii, Nyamira and Gucha receive heavy ranfall whereas the lowland areas around Lake Victoria receive less rainfall. The rainy periods are April/May and October/November. The region is endowed with abundant natural and human resources. The natural resources include the rich soils of Kisii highlands as well as ample rainfall which support agriculture and livestock. Lake Victoria has about 54,000 fishermen operating 11,235 fishing boats. Over 90 % of the country's

total output of fish and products come from Lake Victoria. A number of major rivers traverse the province and drain towards Lake Victoria for example River Nyando, Sondu Miriu and Yala. Most of these rivers are potential for irrigation. Lake Victoria multi-species fisheries are an important economic resource in the region. The agro-based industries are tea processing, coffee, sugarcane and tobacco industries (FAO, 1996).

CHAPTER 2

LITERATURE REVIEW

2.1 Essential oils

Essential oils are volatile natural plant products generally present in low concentrations in specialized structures such as oil cells, glandular trichomes and/or resin ducts (Simon, 1990). They can be distinguished from the fatty vegetable oils such as canola and sunflower by the fact that they evaporate or volatilize with the air and they usually possess a strong aroma. These products are complex mixtures of organic chemicals, the nature and relative proportions of which are primarily determined by the genetics of the plant species. The purity of an essential oil can be determined by its chemical constituents. Variables that may affect these constituents include environmental factors (for example climate and altitude), agricultural factors (such as soil conditions, nutrition, time, harvest conditions and methods of post harvest handling) and process of extraction (Fridge, 2004).

It is necessary to extract or to isolate the essential oil as completely as possible from the mass of inert cellular matter with the minimum amount of chemical change. This may be achieved by several techniques depending on the nature of the starting material. For an extract to be classified as an essential oil, only heat and water may be used in its extraction from the plant (Lucchesi *et al*., 2004). More than 250 types of essential oils are traded in the world market and many developing countries import large quantities of oils to meet local demand for use in soaps, detergents and perfumes.

2.2 Chemistry of essential oils

The chemicals present in essential oils are synthesized during normal development of the herbs and may be classified as hydrocarbons built from multiple of 5-carbon hemi-terpenoid units, oxygenated derivatives of these hydrocarbons, aromatic compounds having a benzoid structure and compounds containing sulphur or nitrogen (Fridge, 2004). Chemically, the essential oils are composed of terpenoids and aromatic polypropanoids synthesized via the melvalonic acid pathway for terpenes and the shikimic acid pathway for aromatic polypropanoids (Simon, 1990). These pathways are schematically represented in Figure 7 (Ebbs, 2005).

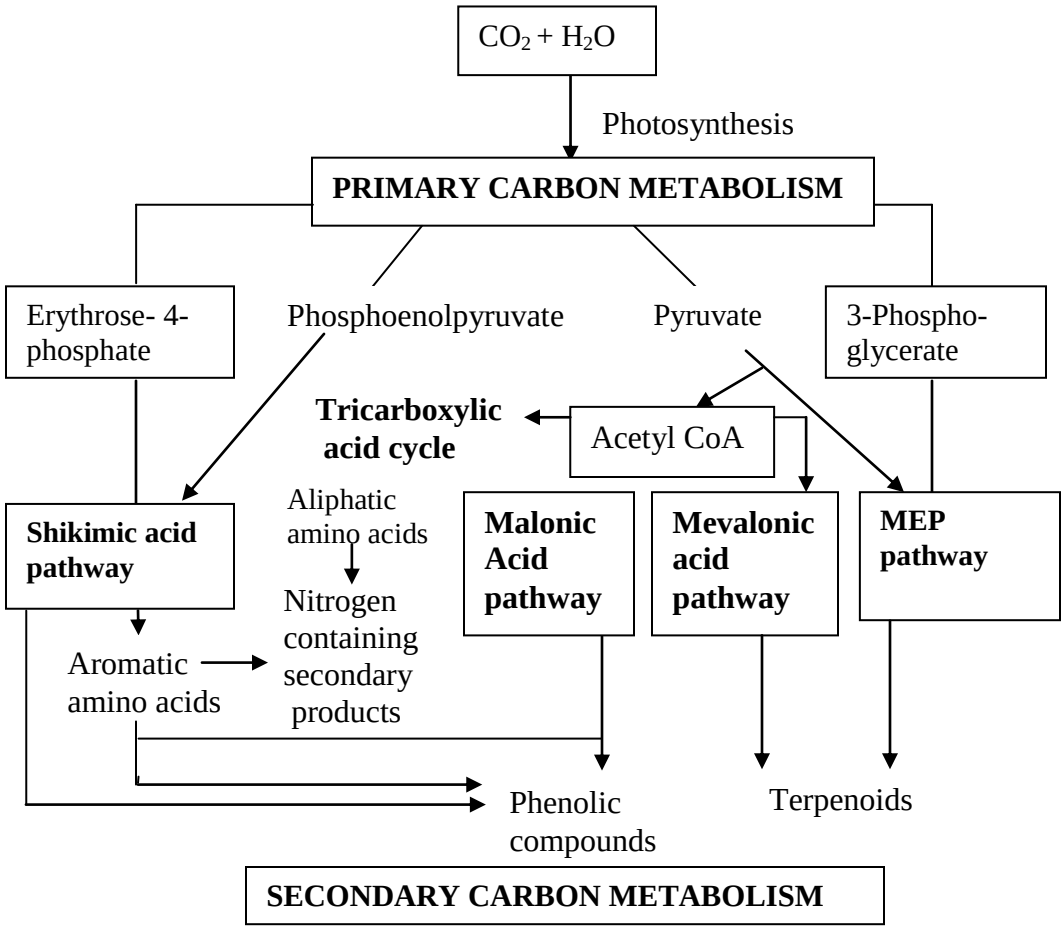


Figure 7: Metabolic pathways for terpenoids and aromatic polypropanoids

Plants have been estimated to collectively synthesize more than 30,000 different terpenoids which constitute the largest family of natural products exceeding in number the alkaloids and phenylpropanoids combined (Simon, 1990). Terpenoids have many useful applications in the manufacture of foods, industrial compounds and pharmaceuticals. They are synthesized from the condensation in a head to tail fashion of 5-carbon isoprene (or hemiterpene) units. Major terpenoid classes include mono-, sesqui- and di-terpenoids, which are mostly secondary metabolites as well as tri- and tetraterpenoids, which are generally primary metabolites (Broun and Somerville, 2001). However, the vast majority are secondary metabolites, including the volatile constituents of essential oils. Monoterpenoids are the primary constituents of many essential oils.

Until recently, it was thought that the synthesis of terpenoids in higher plants was by a cytosolic route that is derived from mevalonate. However, during the last ten years it has become clear that plants also use a parallel plastid pathway that converts pyruvate and glyceraldehydes-3-phosphate to 1-deoxyxyulose-5-phosphate (DXP) which is metabolized in a series of steps to isopentenyl diphosphate and dimethylallyl diphosphate, the common precursors of all terpenoids (Figure 7). Plants use the mevalonate-dependent pathway to synthesize sesquiterpenes and triterpenes whereas other major terpenoids are derived from the 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) pathway. Because discovery of the plastidial route in plants is relatively recent, little is known of the mechanisms that limit flux through the DXPS pathway (Broun and Somerville, 2001).

2.3 Ethnobotanical remedies/practices on tick control and management

An ethnobotanical remedy covers the people's skills, knowledge, methods, practices and beliefs on how to keep animals healthy and it varies across communities (McCorkle *et al*., 1996). Synthetic acaricides present a major drawback as residues in milk and meat, and in the environment where they accumulate and transform into poisonous substances that are toxic to both man and animals (Laffont *et al*., 2001). Consequently, they indiscriminately kill beneficial insects such as bees, birds and have harmful effect on food chains. The acaricides are also expensive to resource-limited farmers; as a result, farmers have reverted back to ethno-botanical remedies (Laffont *et al*., 2001). Ethnobotanical remedies are gaining importance in the management and control of ticks and tick-borne diseases in most African countries (Njoroge and Bussman, 2006). Enthobotanical knowledge offers a range of herbs, some with constituents that are known to possess insecticidal, growth inhibiting, anti-molting and repellent activities. This has stimulated research on their chemical compositions, efficacy and safety, which is likely to promote their effective use by farmers and pastoral communities (Njoroge and Bussman, 2006).

Before World War I and the emergence of synthetic chemical repellents, arthropod repellents were primarily plant-based (Gerberg *et al*., 2007) with oil of citronella being the most widely used compound and standard against which others were tested (Dethier, 1956). Use of the early synthetic repellents such as dimethyl phthalate (DMP) which was discovered in 1929, indalone (butyl-3,3-dihydro-2,2-dimethyl-4-oxo-2H-pyran-6-carboxylate) which was secured in 1937, and ethyl hexanediol which was made available

in 1939, was overshadowed by the discovery of *N*,*N*-diethyl-3-methylbenzamide (DEET) which gradually became the gold standard for arthropod repellents (Strickman, 2007). Over 20,000 compounds have been screened for repellency against arthropods, yet none have resulted in a product of equal commercial success to that of DEET with its broad-spectrum range of protection and duration of repellency (Gupta *et al*., 2007). DEET was formulated as an arthropod repellent in 1946 (Xue *et al*., 2007) and registered for commercial use in 1957. DEET is the active ingredient in the majority of commercially available tick repellents used on human skin today and is effective against several tick and mosquito species (Schreck *et al*., 1995).

DEET has been the most extensively used as a chemical substance that cause anthropods to make oriented movements away from it (Dethier *et al*., 1960). DEET has also been used as a personal arthropod repellent for over 5 decades and is available in a wide range of concentrations and products that can be applied to exposed skin or clothing (Frances, 2007a). However, when the repellent is applied to human, it causes allergic reactions, seizures, mood disturbances, insomnia and impaired cognitive functions (Robbins and Cherniack, 1986; Lewis *et al*., 2000). DEET also causes considerable environmental pollution (Seo *et al*., 2005). DEET has an ability to dissolve synthetic materials such as plastics, spandex, leather and fabrics and this has led to search for safe and alternative repellents for arthropods (Seo *et al*., 2005).

Ethnobotanical remedies provide valuable alternatives to compliment the chemical acaricides especially where they are not affordable, available and appropriate to use. It is

an approach that offers sustainable strategies directed towards developing sound and appropriate animal health care systems suitable and relevant to rural communities in improving livestock performance and production (Wanzala *et al*., 2005). Studies have shown that some plants and their products are comparable to DEET as repellents and can be even better (Panda, 2004; Chauhan *et al*., 2005; Bond, 2007).

Most plants are used as tick repellents because they have been found to exhibit acaricidal and/or tick repellent properties. They include cat's whiskers plant (*Cleome gynandra* L.) (Pandey *et al*., 1983a, 1983b; Chandel *et al*., 1987; Verma and Pandey, 1987; Malonza *et al*., 1992), *Melinis minutiflora* P. Beauv (Thompson *et al*., 1978; Mwangi *et al*., 1995a), *Stylosanthes scabra* Vog. (Sutherst *et al*., 1982; Zimmerman *et al*., 1984; Wilson *et al*., 1989), *Stylosanthes viscose* (L.) Sw. (Zimmerman *et al*., 1984), *Andropogan gayanus* Kunth (Cruz-Vanquez *et al*., 2000), *Gynandropsis gynandra* (L.) Briq. (Malonza *et al*., 1992), *Acalypha fruticosa* Forssk (Hassan *et al*., 1994), *Ptaeroxylon obliquum* (sneeeze wood) (Archer and Reynolds, 2001), *Azadirachta indica* A. Juss. (neem) (Williams, 1993; Kalakumar *et al*., 2000; Benavids *et a*l., 2001; Webb and David, 2002), 'Kupetaba' (tobacco leaves and Magadi soda mixture) (Dipeolu and Ndungu, 1991), *Margaritaria discoidea* (Kaaya *et al*., 1995), *Ocimum suave* Willd (Mwangi *et al*., 1995b), *Tithonia diversifolia* (Hemsl.) A. Gray and *T. minuta* (Njoroge and Bussmann, 2006) and *Tephrosa vogelli* Hook. f. (Kaposhi, 1992).

Cat's whiskers plant (*Cleome gynandra*) belongs to the botanical family Capparaceae. It is an erect herbaceous annual herb, which is branched and rather stout. Depending on

environmental conditions, it can grow up to 1.5 m in height, and is usually 0.5-1.0 m tall. *C. gynandra* has been observed to have insecticidal, antifeedant and repellent characteristics (Verma and Pandey 1987; Pandey *et al*., 1983a, 1983b; Chandel *et al*., 1987; Malonza *et al*., 1992). The leaves have anti-tick properties. They also have repellent and acaricidal properties for the larvae, nymphs and adult *R. appendiculatus* and *A. variegatum* ticks. Ticks are not found for a distance of 2-5 m from the plant.

Melinis minutiflora is a species of grass commonly known as molasses grass and belongs to the family of Poaceae. The whole plant is insecticidal and has been cultivated in Brazil and central Africa for this purpose (Watt and Breyer-Brandwijk, 1962). Of six grass species investigated, molasses grass showed the highest anti-tick properties. *Andropogan gayanus* had the ability to maintain a defined, constantly low, initial host tick infestation and lengthy but low to moderate field tick population (Cruz-Vanquez *et al*., 2000). The lethal and repellent effect of *M. minutiflora* grown on monoculture plots against the larvae of *B. microplus* infestations have been previously studied in the work of de Barros and Evans (1989) and the grass has been considered of potential use for biological control of cattle tick. Leaves and stems of this graminoid are covered with trichomes (or glandular hairs) where a viscous fluid of characteristic odor is secreted. This oily material is reported to be responsible for the ability of molasses grass to reduce cattle-tick infestation either by repelling or killing tick larvae. It is insecticidal, arachnicidal and an insect repellent (Corrêa *et al*., 1952). *Melinis minutiflora* is a species which would best be used in tick control within a marginal tick zone, while *A. gayanus* would be better within an endemic tick zone (Thompson *et al*., 1978).

Studies conducted by Hernandez *et al*. (1990) and Mwangi *et al*. (1995) demonstrated that the molasses grass repels ticks (*R. appendiculatus* and *B. microplus*). They conducted *in vitro* studies on adults, nymphs and larvae and concluded from olfactometer studies that the repellent effect was a result of a strong volatile chemical. Also the long trichomes on the stems and leaves of the grass also impede the movement of larvae upwards. In field experiments with cattle-tick, molasses grass indicated repellent rather than larvicidal properties (Menendenz, 1924). The lethal effect upon larvae was explained by Jesus (1934) as being a consequence of exhaustion resulting from mechanical effect caused by a viscous secretion of the plant which prevented the larvae from climbing the stem and reaching the leaves and asphyxiation when body of larvae becomes covered by the plant secretion. A severe reduction in larval (Thompson *et al*., 1978) and adult (Aycardi *et al*., 1984) tick population induced by molasses grass has been described. *In vitro* and *in vivo* assays (Alvarez *et al*., 1986) permitted identification of the biological activity of molasses grass as being due to the trichomal essential oil, which is actually responsible for the repellent, acaricidal and ovicidal properties.

Stylosanthes spp are grass species that possess anti-tick activities and they belong to the family of Fabaceae. They are highly nutritious tropical legumes covered in glandular hairs (trichomes) that secrete a viscous fluid capable of entrapping ticks (Sutherst *et al*., 1982). The effect of *S. scabra* and *S. viscose* on the larvae of *B. microplus* has been reported (Sutherst *et al*., 1982). It was shown that the larvae were trapped and immobilized in the glandular trichomes of greenhouse reared, potted plants and killed rapidly by an unknown toxin contained in the viscous secretion of the trichomes thus the

larvae did not proceed to the tips of the plants. The utility of the *Stylosanthes spp* was very high due to its persistence and high productivity in the tropics, improvement of cattle nutrition, which should in turn improve their natural resistance to ticks, and, being leguminous plants, they also improve soil fertility through nitrogen fixation.

Further studies of *S. scabra* and *S. viscose* in Puerto Rico confirmed that *Stylosanthes* species yielded the fewest live larvae of *B. microplus* and the larvae and nymphs of *A. variegatum* (Zimmerman *et al*., 1984). Also, Norval and his co-workers suggested that *Stylosanthes spp* repelled the larvae as they preferred to climb wheat straw when given choice between them and *Stylosanthes spp* (Norval *et al*., 1985). Also the anti-tick effect may be enhanced by the observed accumulation of the secretions, especially of *S. viscosa* on the legs and heads of grazing cattle (Sutherst *et al*., 1982). A high density of a non-glandular fine hairs also prevent larvae from ascending these stems (Sutherst *et al*., 1982: Sutherst *et al*., 1988)

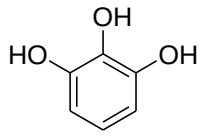
Gynandropsis gynandra is a shrub that belongs to the family of Capparidaceae. It grows as a weed in most parts of the world and is mostly found in fields grazed by cattle (Malonza *et al*., 1992). *In vitro* studies of *G. gynandra* show that the whole plant material has acaricidal and repellent properties on ticks (Lwande *et al*., 1999; Songsak and Lockwood, 2002). A study of *G. gynandra* showed that it has repellent and acaricidal properties to *R. appendiculatus* and *A. variegatum* ticks of all active stages (Malonza *et al*., 1992; Ndungu *et al*., 1995). In grazing paddocks where the plant was present in high numbers it was found that there was a total absence of ticks as compared to the

neighboring paddocks where *G. gynandra* was absent (Malonza *et al*., 1992). Also the oil extract of *G. gynandra* have been tested and found to have a repellent effect against *R. appendiculatus* ticks (Lwande *et al*., 1999; Songsak and Lockwood, 2002).

Acalypha fruticosa is a species in the botanical family of Euphorbiaceae. It is unpalatable aromatic shrub commonly found in grazing zones of Kenya. It is valued in India and Africa for its medicinal properties (Alasbahi *et al*., 1999; Nadanakunjidam, 2003). The leaves of the *A. fruticosa* attracted the larvae particularly those of *R. appendiculatus* in the field and *in vitro* (Hassan *et al*., 1994). In the bush surrounding the grazing sites, it was noted that the ticks were consistently found on the underside of the leaves of the plant. As a result, it was recommended that the plant can be planted along the grazing pastures to attract the ticks and they could be controlled by selectively destroying the leaves infested by the ticks (Hassan *et al*., 1994).

Ptaeroxylon obliquum (Sneeeze wood) is a species of plant that belongs to the Rutaceae family. Rutaceae are most abundant in South Africa and Australia. The tree is used traditionally both for medicine and ritual purposes. Bark is used as a snuff to relieve headaches. The strong and durable wood contains an aromatic resin that causes violent sneezing. Pieces of wood can be placed in cupboards to act as moth repellent. The bark of *P. obliquum* has been reported to contain; pyrogallol (**4**), resins and an alkaloid (Archer and Reynolds, 2001). The aromatic plant resins were reported to possess essential oils which had acaricidal and repellency activities (Pontes *et al*., 2007). The resin from the heated wood has been applied to warts and powdered bark added to a wash to kill cattle

ticks (Archer and Reynolds, 2001). The aged resin oil induced more tick repellency compared to the fresh one (Pontes *et al*., 2007).



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Azadirachta indica (Neem) is a tree in the mahogany family Meliaceae. Oil from seeds of the *A. indica* tree has shown to have inhibitory effects on vitellogenin during oogenesis of arthropods (Williams, 1993). Several studies on the effect of neem extracts on cattle ticks have been conducted on different tick species such as *A. hebraeum*, *Rhipicephalus evertsi* Neuman, 1987, *H. truncatum* and *R. (B) decoloratus* and found that in overall it reduced tick load burden in cattle significantly (Williams, 1993; Kalakumar *et al*., 2000; Benavids *et al*., 2001; Webb and David, 2002). Undiluted neem oil containing azadirachtin was found to be effective against *R. appendiculatus, A. variegatum* and *B. decoloratus* (Kaaya and Saxena, 1998). The oil was applied directly on ticks attached to animals (rabbits and cattle), and in rabbits, it was effective against all tick species. The oil deterred larval and nymphal attachment, inhibited feeding, reduced fecundity and egg hatchability, molting of the larvae and nymphs. Ethanol extracts of neem were found to be effective at inhibiting oviposition (Williams, 1993).

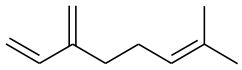
Tobacco and Magadi soda mixture (‘Kupetaba’**)** is ground mixture of dried tobacco leaves and Magadi soda mineral, which is mined around Lake Magadi in the Rift Valley Province of Kenya. The *in vitro* and *in vivo* effects of ‘Kupetaba’ were evaluated and

proved to be effective as an acaricide against all stages of *R. appendiculatus* (Dipeolu and Ndungu, 1991). The substance prevented the completion of all feeding phases of the tick, suppressed the oviposition capacity of the engorged ticks and drastically reduced the hatchability of eggs. Larvae and nymphs were killed within 24 hours of the application of the substance on calves' ears and large numbers of adult ticks were also killed within 2–3 days of application during *in vitro* experiments (Dipeolu and Ndungu, 1991). 'Kupetaba' may readily be adapted by farmers for use within an integrated tick management strategy for a number of reasons. It is cheap and affordable, non-poisonous to cattle since no harmful effects were observed on any experimental cattle during this investigation and it is easy to prepare (Dipeolu and Ndungu, 1991).

Margaritaria discoidea is a termite-resistant tree that belongs to the family of Euphorbiaceae. It grows to about 10 m along riverbanks in Africa. Its wood is used as a perfume and is burnt as a mosquito and snake repellent (Kaaya *et al*., 1995). Aqueous extracts of bark and hexane extracts of wood were prepared and mortality studies were undertaken on *A. variegatum* and *R. appendiculatus* ticks (Kaaya *et al*., 1995). The authors conducted *in vitro* repellency and dose mortality studies as well as *in vivo* controlled studies using rabbits and cattle. Residual activity was also examined. Water extracts of bark were variably effective against nymphs of both species. Hexane oil extracts from the wood appeared to have a stronger acaricidal effect *in vitro* than the aqueous extracts of bark, with almost all concentrations resulting in 100 percent mortality. *In vitro* repellency studies indicated a strong effect against *R. appendiculatus* adults and nymphs. 50 percent hexane extract of *M. discoidea* in corn oil resulted in 90

and 100 percent reductions of ticks in zebu cattle that were naturally infested with *R. appendiculatus* (Kaaya *et al*., 1995).

Ocimum suave is a shrub that belongs to the family of Labiatae and is commonly found in the upland areas of East Africa. It has been used as an insect repellent and for a variety of medicinal purposes (Mwangi *et al*., 1995b). Dose mortality studies using oil extracted from the plant by steam distillation were conducted on the larvae of *R. appendiculatus* and demonstrated a high level of effectiveness (Mwangi *et al*., 1995b). The oil was also found to be repellent *in vitro* and *in vivo* studies showed that the oil could protect rabbits from larval infestation. Also, essential oil constituents have been used as tick repellents. (Ibrahim *et al*., 2001). Myrcene (**5**) and 4-terpineol (**6**) are repellent to *R. appendiculatus* (Ndungu *et al*., 1995).



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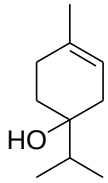

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Tithonia diversifolia commonly known as Mexican sunflower was probably introduced into West Africa as an ornamental plant (Akobundu *et al*., 1987). It belongs to the family Asteraceae. *T. diversifolia* is an annual, aggressive weed growing to a height of about 2.5 m and adaptable to most soils. It had been observed to be widely spread in Nigeria where it is found growing on abandoned/waste lands, along major roads, waterways and on cultivated farmlands. In Kenya, *T. diversifolia* and *T. minuta* have been reported to have anti-tick repellent properties against *R. appendiculatus* (Njoroge

and Bussmann, 2006; Wanzala, 2009). Also, resource-limited farmers are reported to mix 100 ml of nicotine with about 1 liter of used engine oil to make oil dressing to kill ticks (Forse, 1999). Despite the use of Jeyes fluid and used engine oil as acaricide, they have some fatal detrimental effects both on animals and the environment. Used engine oil contains heavy metals such as cadmium, arsenic and lead which can be toxic (Turkson, 2001; Villarino *et al*., 2003).

The integration of tick repellent plants and their products with other tick control remedies on the host and in the grazing field would be a practical and an economical way of controlling ticks and other ectoparasites. Since these results reflected low knowledge on plant use, there is an urgent need to study scientifically the enthnobotanical practices, document and validate information on use of ethno-veterinary remedies as acaricides for the benefit of the new generation and the resource-limited farmers so that the knowledge can be preserved, plants conserved and sustainably managed.

2.4 *Tagetes minuta* L.

2.4.1 Origin and morphology of *T. minuta*

Tagetes minuta is a marigold species (Figure 8) from the genus *Tagetes* and it belongs to the family of Asteraceae commonly known as Mexican or wild marigold (English), Omosumo (Kisii), Abuba (Luo), Mũbangi (Kikuyu), Ngwekwe (Kibukusu) and Chemiasoriet (Kalenjin). It is native to the southern half of South America, and ever since Spanish colonization; it has been introduced around the world, including Europe, Asia, and Africa. *T. minuta* is an erect annual herb reaching 1 to 2 m. Leaves are slightly glossy

green, and are pinnately dissected into 4 to 6 pairs of pinnae. Leaf margins are finely serrate. There are typically 3 to 5 yellow-orange ray florets, and 10 to 15 yellow-orange disk florets per capitula. The undersurface of the leaves bear a number of small, punctuate, multicellular glands, orangish in colour, which exudates a licorice-like aroma when ruptured (Prakaso *et al*., 1999). The heads are small, 10 to 15 mm long, and including ray florets, 10 to 20 mm in diameter. Wild marigold as it is commonly known is a problematic weed of pastures and numerous crops in East and South Africa, South America, and Australia (Soule, 1993).

Figure 8: Aerial parts with flowers of *T. minuta*

2.4.2 Ecology of *T. minuta*

Tagetes minuta is native to temperate grasslands and montane regions of southern south of America (McVangh, 1943) including Chile (Reiche, 1903) and Bolivia (Perkins, 1912). During the Spanish colonization of South America, it was introduced into Africa, Asia and Australia. *T. minuta* has become a major agricultural weed in 35 countries (Jauzein, 1995). Wild marigold is a problematic weed of pastures and numerous crops in East and South Africa, South America and Australia. The seed has unpleasant odor and can reduce the value of grain harvests when it is a contaminant (Nada, 2008). Root extracts are allepathic to many vegetables, corn and sunflower. Wild marigold is resistant to natural enemies (Nada, 2008). Due to robust habitus, it is a fierce competitor for space and light. Once established and uncontrolled it creates dense monotypic stands and displaces other plants. The plant usually prefers to grow on light (sandy), medium (loamy) and heavy (clay) soils which are well-drained, acidic, neutral and alkaline. It cannot grow in the shade and prefers cultivated beds.

2.4.3 Commercial and enthobotanic uses of *T. minuta*

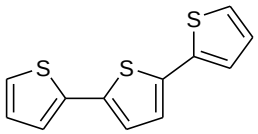
Tagetes minuta is commercially grown and harvested for its essential oil which is used in the flavor and perfume industry as *Tagetes* oil (Lawrence, 1993). The oil is used in perfumes and as a flavor component in most major food products including cola beverages, alcoholic beverages, frozen dairy desserts, candy, baked goods, gelatins, puddings, condiments, and relishes (Leung, 1980). An essential oil obtained from the distilled plant harvested when in flower, is used for flavoring ice cream, baked foods, and soft drinks (Leung, 1980).

For medicinal use, a decoction made by steeping a double handful of the dried plant in boiling water for 3 to 5 minutes is used as a remedy for the common cold, including upper and lower respiratory tract inflammations, and for digestive system complaints, stomach upset, diarrhea, and liver ailments. The decoction is consumed warm, and may be sweetened to individual taste (Parodi, 1959; Neher, 1968). The plant is popularly used as anthelmatic, diuretic, antispasmodic and to treat stomach and intestinal diseases in native regions of the world (Amat, 1983). It can also be used as a medicine to treat diseases such as Athlete's foot, chest infections and cough cataract (Morton, 1981). In Kenya, an infusion *T. minuta* is used for treatment of snake bites in the Kamba and Luo communities (Owuor and Kisangau, 2006) and protection against mosquito bites by communities of western Kenya (Seyoum *et al*., 2002).

2.4.4 Bioactive properties of *T. minuta*

Wild marigold has been widely cultivated around the world due to its agrochemical and pharmacological properties (Nada, 2008). It is very effective biopesticide and root secretion kills subsurface and surface soil pathogens (Héthélyi *et al*., 1986a; Nada, 2008). The plant has suppressive effect on free-living nematodes and has been used as an intercrop in rotation to protect crops (Kimpinski and Arsenault, 1994). Floral, foliar and root extracts have insecticidal activity against adult *Coleoptera* and mosquito larvae and adults (Keita *et al*., 2000; Sarin, 2004; Seyoum *et al*., 2002). Its root extracts can reduce populations of the weed species *Agropyron repens* and *Convolvulus arvensis* (Nada, 2008). In Africa, it is usually grown by organic gardeners for its insecticidal and nematocidal activities between lettuce, cabbage and tomatoes (Krueger, 2007; Nada, 2008). Mexican marigold spray formed by crushing large quantities of fresh flowers and

leaves, in a bucket of water, left for 5 to 7 days and daily stirred is used as a preventive measure for pests and diseases in crops (Soule, 1993). The spray is applied using spraying equipment but also the solution can be sprinkled using twigs or grass tied together to form a whisk. The spray is effective when the weather is not too damp and is applied once a week (Nada, 2008). It is applied on coffee to prevent coffee berry disease and protects peas from blight, mildew and other fungal diseases because it produces a substance called alpha-terthienyl (**7**) which can aid in the reduction of disease promoting organisms such as fungi, bacteria, insects, and some viruses (Héthélyi *et al*., 1986a; Soule, 1993). It also repels aphids, termites, blowflies, caterpillars, ants, maggots, flies and moths.



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It has been reported that the secondary compounds in *Tagetes* are effective deterrents of numerous organisms including fungi (Chan *et al*., 1975), bacteria (Grover and Rao, 1978), round worms (Loewe, 1974), trematodes (Graham *et al*., 1980), nematodes (Grainge and Ahmed, 1988), and other numerous insect pests (Jacobsen, 1990). *T. minuta* also exhibits insecticidal activity against stored products pests (Keita *et al*., 2000; Sarin, 2004) and mosquitoes (Seyoum *et al*., 2002). It has also been reported that control of root knot nematodes in rose flower beds with *T. minuta* extract is as effective as furadan, nemacure and temik insecticides. Rose plants with feeder roots destroyed by root knot

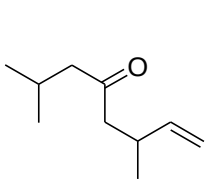
nematodes showed significant recovery one month after treatment of the soil with an extract from *T. minuta* (Goswani, 1986).

2.4.5 Chemotypes of *T. minuta*

The composition of the essential oils of *T. minuta* from different countries has been reported previously (Machado *et al*., 1994; Lawrence, 2000; Stojanova *et al*., 2000) and showed significant differences in their composition (Lawrence, 1985b; Graven *et al*., 1991). The analyses of essential oils of chemotypes (genetic and epigenetic) of *T. minuta* (Gil *et al*., 2000) indicates that the composition of the oils varies according to a number of factors: the harvesting location (Cravelro *et al*., 1988; Chalchat *et al*., 1995), stage of harvest (Héthélyi *et al*., 1986b), plant parts distilled (Héthélyi *et al*., 1986a), soil type and nutrient status (Graven *et al*., 1991) and the climatic condition under which the plant grows (Mohamed *et al*., 2002). *T. minuta* is rich in secondary compounds such as acyclic, monocyclic and bicyclic monoterpenoids and sesquiterpenoids, and flavonoids, thiophenes, and aromatics (Rodriguez and Mabry, 1977).

In Argentina GC and GC-MS analyses of the essential oil of *T. minuta* showed that dihydrotagetone (**8**), α-phellandrene (**9**), limonene (**10**), *o*-cymene (**11**), β-ocimene (**12**), *trans*-tagetone (**13**) and tagetenone (**14**) were the major constituents (Gil *et al*., 2000). In Eygpt, the main constituents of *T. minuta* essential oil were monoterpenes of which *trans*- and *cis*-tagetone (**15**) were present in 52.3 % and 64.2 % respectively (Mohamed *et al*., 2002). In South Africa, GC-MS analyses of the essential oil of *T. minuta* showed that β-ocimene (32.0 %) and dihdrotagetone (16.4 %) were the main components (Mohamed

et al., 2004). In south west of Iran, the analyses of the chemical composition of essential oil of *T. minuta* showed that the oil was particularly rich in limonene (13.0 %), piperitenone (**16**) (12.2 %), α-terpinolene (**17**) (11.0 %), piperitone (**18**) (6 %), *cis*-tagetone (5.7 %), (Z) ocimenone (**19**) (5.1 %) and eucarvone (**20**) (4.8 %) (Mohammad *et al*., 2010).



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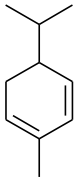

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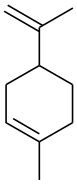

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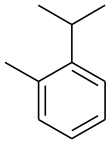

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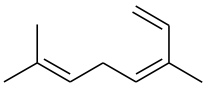

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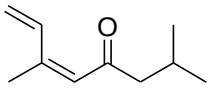

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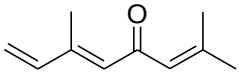

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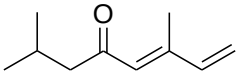

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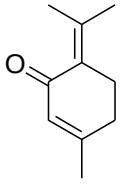

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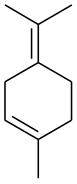

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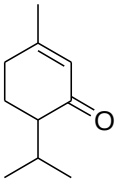

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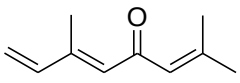

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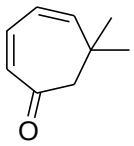

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2.4.6 Significance of chemotypic differences in plant exploitation

A chemotype is largely an epigenetic (with some genetic) variation of a plant caused by the effects of sunlight, soil, temperature and weather conditions (Mohamed *et al*., 2002). Botanically, the plants are identical but their chemical compositions are different. Different chemotypes of the same essential oil sometimes have different effects when used in aromatherapy. Users of essential oils can use chemotypes to create interesting variations in personal fragrances, cosmetics and aromatherapy (Lawrence, 1993).

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant materials

Tagetes minuta was sampled from three different agro-ecological zones: Nairobi-Kasarani, western-Bungoma and Nyanza-Bondo. From each zone, *T. minuta* was sampled from five different sites and a total of 100 plants were harvested from each site when the plant was at flowering stage in September, 2009. Samples of the plant were identified by a taxonomist, Mr. K. Gichovi, in the Department of Plant and Microbial Sciences, Kenyatta University and the specimen voucher no. MOB/06/09/2009 deposited in Kenyatta University museum.

3.2 Isolation of essential oils

The plant materials were dried in a well ventilated room under shade for 1 week. The dried plant materials were cut into small pieces and 500 g of each plant was placed in a 2 liter round-bottomed flask containing 1.5 liters of water and then hydrodistilled using a Clevenger-type apparatus for 8 hours (Clevenger, 1928). Pure oil samples from different plant collections were collected from the plants into 2 ml vials and stored at −20 ^{0}C in a freezer until required for analysis and bioassays.

3.3 Experimental ticks

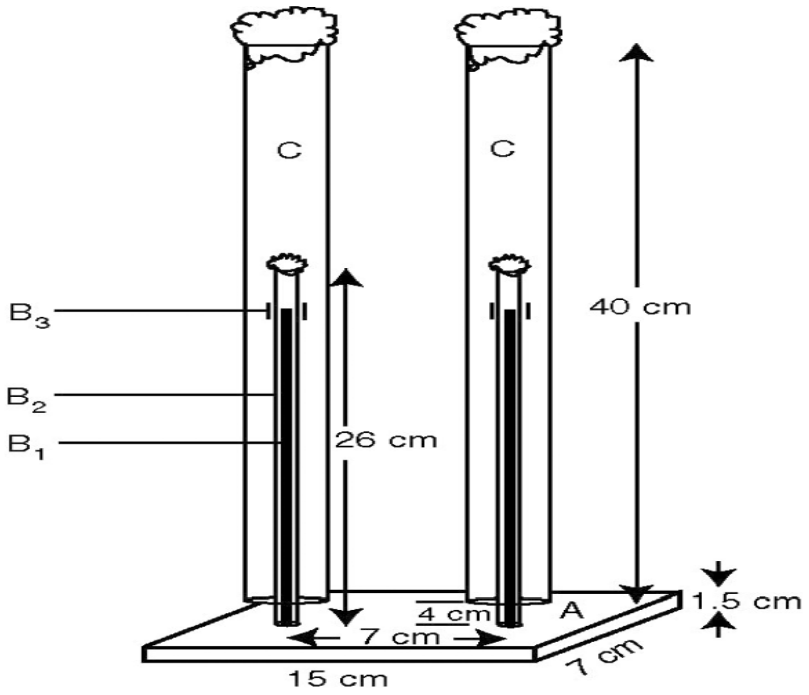
The ticks (*R. appendiculatus*) used were obtained from colonies reared in the insectary of the International Centre of Insects Physiology and Ecology (ICIPE), Nairobi, Kenya. All bioassays were conducted with the newly-emerged adults.

3.4 Tick climbing assay

A dual-choice tick climbing assay apparatus was used for screening the repellency of (i) the essential oils of *T. minuta* obtained from three different agro-ecological zones, (ii) individual standard constituents obtained from Sigma Aldrich Company, and (iii) different blends of these constituents all at concentrations of 0.5, 1 and 2 mg/ml. Subtraction bioassay method was used to remove one component from the blend at a time so as to check the dose response of the remaining blend against *R. appendiculatus*. The assay apparatus exploited the behavior of the adult ticks, *R. appendiculatus,* which climb up grass stems to the stem tip to wait for any potential passing host (Browning, 1976; Chiera, 1985). The bioassays were done using an improvised tick climbing assay according to the specification set up in the laboratory at ICIPE (Wanzala, 2004). An aluminum base of area 105 cm^2 with two stands of 26 cm in height and 7.0 cm apart will be put in a basin of water, 1.5 cm deep (to retain the ticks at the base) as presented in Figure 9.

A strip of filter paper (Whatmann No 7, 4.25 cm wide) was stapled to form a collar around the upper parts of each smaller inner tube at a distance of 20 cm from the aluminum base to provide the source of either test odors or pure solvent. One collar on the pair of the tubes was treated with test odor solution and the other one with the same

amount of pure solvent (dichloromethane) alone to serve as control. The solvent was allowed to evaporate for about 10 minutes after which these tubes was shielded with wider tubes (4.5 cm diameter) from 4 cm above the aluminum base to shield the inner ones and limit the diffusion of the test material laterally and facilitate relatively uniform vertical gradients of the odors along the 3.7 cm gap between two tubes. The upper ends of the larger tubes were plugged with dry cotton wool. The top of the smaller tubes was plugged with wet cotton wool to ensure relatively high relative humidity (>75 %) within the columns.



A – aluminum base, B_1 – metal rod, B_2 – inner glass tube, B_3 – collar and C – outer glass tube

Figure 9: Tick climbing assay setup

The isolated essential oils of *T. minuta* obtained from three different agro-ecological zones were weighed and dissolved in 1 ml of dichloromethane separately and prepared into different concentrations of 0.5, 1 and 2 mg/ml. The test materials was dispensed by a calibrated Eppendorf pipette equilibrated for 30 minutes and then five adult ticks of mixed ages and sexes were released at the centre of the aluminum base and ten replicates were assayed at each concentration. Before the bioassays, ticks were kept at high relative humidity (RH) (>85 %) for 24 hours in containers with moist cotton wool. All assays were conducted in a room of 28±1 ^{0}C and 75±5 % RH. The assays were left to run for 2 hours during which the number of ticks that climbed on the control glass tube, Nc and on the treated glass tube, Nt were counted and recorded after every fifteen minutes for two hours.

The preliminary results obtained and analysed would not have allowed the data analysis to start at the 15th and 30th minute of observation but start at the 45th minute because this was to allow the ticks to settle and adjust to the environment. After each test the apparatus was thoroughly cleaned and dried at 100 ^{0}C. Initial comparison of the responses of ticks in the set up with and without residual dichloromethane on one and both sides, showed no bias for either side and no effects of the residual solvent. The repellency effect of the essential oils, individual constituents and blends of the more repellent oil from Nairobi-Kasarani zone were evaluated at different doses according to the formula adopted by Ndung'u *et al*. (1995) and Lwande *et al*. (1999); that is, percentage of repellency (PR) = [Nc-Nt]/ [Nc+Nt] × 100.

3.5 Gas chromatography-linked mass spectrometry analyses

The composition of the volatile constituents of the essential oils from *T. minuta* was established by GC-MS. Five drops of the oil samples were dissolved in 1 ml dichloromethane, diluted and analyzed. One microlitre of the oil sample was injected into the HP 6890 series gas chromatography interfaced to a 5973 Mass Selective Detector (MSD) and controlled by HP chemstation software (version b.02.05, 1989-1997). The chromatographic separation was achieved using a DB-5 MS capillary column (30.0 m x 250 m x 0.25 m). The column stationary phase comprised of 5 %-Diphenyl-95 %-dimethylpolysiloxane. The operating GC condition was an initial oven temperature of 50 °C then programmed to 300 °C at the rate of 10 °C/minute and then kept constant at 300 °C for 3 minutes. The injector and detector temperatures were set at 250 °C. The mass spectrometer was operated in the electron impact mode at 70 eV. Ion source and transfer line temperature was kept at 300 °C. The mass spectra were obtained by centroid scan of the mass range from 40 to 800 amu. Identification of the constituents was done by comparing mass spectra with library mass search database (NIST & WILEY).

3.6 Synthetic standards

Guaiacol, β-ocimene and allo-ocimene synthetic standards used in the bioassays were purchased from Sigma Aldrich Company.

3.7 Blends

Blends were prepared from the major constituents of the more repellent essential oil of *T. minuta* obtained from Nairobi-Kasarani zone (guaiacol, β-ocimene and allo-ocimene). They were prepared by mixing the individual constituents based on the ratio of their

relative amounts found from the GC-MS analyses and their repellency tested against *R. appendiculatus* at concentrations of 0.5, 1 and 2 mg/ml. These blends were OGA (mixture of β-ocimene, guaiacol and allo-ocimene, 13:13:10), OG (mixture of β-ocimene and guaiacol, 13:13), GA (mixture of guaiacol and allo-ocimene, 13:10) and OA (mixture of β-ocimene and allo-ocimene, 13:10).

3.8 Data analyses

The data obtained was transferred into statistical products and service solutions spreadsheet database and analyzed. The repellency data obtained at different doses was subjected to analysis of variance (ANOVA) for a completely randomized design. Treatment means showing significant difference ($p \leq 0.05$) were separated using Student-Newman-Keuls (SNK) at the 5% significance level. Dose-response relationship was determined using probit analysis and repellent doses at RD_{50} and RD_{75} values obtained from regression model.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Yield and physical characteristics of the essential oils

Percentage essential oil samples obtained from replicate dried aerial parts (500 g) of *T. minuta* originating from the same agro-ecological zone were similar. However, the yields of the essential oils from plant samples from the three different zones were different. Aerial parts sampled from Nairobi-Kasarani zone produced the highest percentage of the essential oil of 0.045±0.005 % w/w while those obtained from Nyanza-bondo and western-Bungoma zones produced a yield of 0.035±0.005 and 0.039±0.005 % w/w, respectively. The essential oils of *T. minuta* collected were less dense than water and exhibited a pale yellow color with a turpentine-like odor (El Deeb *et al*., 2004). The oils remained liquid at room temperature and were insoluble in water but soluble in ethanol, ether and dichloromethane (El Deeb *et al*., 2004).

4.2 Composition of the essential oils of *T. minuta* from different zones

GC-MS analyses revealed that each sample of essential oil of *T. minuta* contained more than 45 constituent compounds. The compounds and their relative proportions in the essential oils from the three zones are given in Appendices 1a, 1b and 1c. However, GC-MS analyses of the essential oils showed that samples from the same agro-ecological zone were more similar in relative amounts but varied in the three zones. The major constituents and their relative abundances characterized are listed in Table 1. The essential oils comprised of hydrocarbons mainly terpenes (monoterpenes, sesquiterpenes

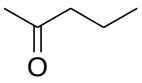
and diterpenes), oxygenated terpenes, alcohols, aldehydes, carboxylic acids, esters, fatty acids, nitrogenated terpenes and ketones. About 30 % (3 compounds) of essential oil from plants growing in Nairobi-Kasarani zone were monoterpene hydrocarbons while the rest ~70 % (7 compounds) were sesquiterpene hydrocarbons. The samples from plants collected from western-Bungoma zone were made up 20 % (2 compounds) monoterpene hydrocarbons, 10 % (1 compound) chlorinated sesquiterpene, and 70 % (7 compounds) sesquiterpene hydrocarbons. The samples from plants originating from Nyanza-Bondo zone were found to comprise of 40 % (4 compounds) monoterpene hydrocarbons, 50 % (5 compounds) sesquiterpene hydrocarbons, and 10 % (1 compound) nitrogenated sesquiterpene.

Table 1: GC-MS major constituents identified in the essential oils of *T. minuta* obtained from three different agro-ecological zones.

No.	Compound	Molecular formula	M^+ (g/mol)	Relative (%)		
				Nairobi-Kasarani	Western-Bungoma	Nyanza-Bondo
1.	β-Ocimene	$C_{10}H_{16}$	136.2	13.2	3.8	–
2.	Limonene	$C_{10}H_{16}$	136.2	4.3	3.3	4.8
3.	Dihydrotagetone	$C_{10}H_{18}O$	154.0	8.2	8.7	9.5
4.	Allo-ocimene	$C_{10}H_{16}$	136.2	10.2	13.9	–
5.	*cis*-Tagetone	$C_{10}H_{16}O$	152.2	2.9	–	–
6.	*trans*-Tagetone	$C_{10}H_{16}O$	152.2	9.4	4.6	–
7.	β-Ocimenone	$C_{10}H_{14}O$	150.2	6.3	5.2	–
8.	Guaiacol	$C_7H_8O_2$	124.1	13.1	–	–
9.	Car-3-en-2-one	$C_{10}H_{14}O$	150.2	4.1	–	–
10.	Eucarvone	$C_{10}H_{14}O$	150.2	3.6	–	–
11.	2-Pentanone	$C_5H_{10}O$	86.1		25.3	–
12	Ether,2-chloro-1- methylethyl isopropyl	$C_6H_{12}Cl_2O$	171.2	–	3.8	–
13.	2-Hexanone	$C_6H_{12}O$	100.0	–	4.1	–
14.	2-Cyclohexen-1-ol-3,5,5-trimethyl	$C_9H_{16}O$	140.3	–	3.6	2.2
15.	1-(1-Hydroxy-1-methyl(-ethyl)-cyclobutane carboxylic acid	$C_8H_{17}O_3$	161.2	–	–	2.3
16.	Morpholine,4[3-(4-butoxy-phenoxy) propyl]-	$C_{17}H_{27}NO_3$	273.2	–	–	2.3
17.	Modheph-2-ene	$C_{15}H_{24}$	204.3	–	–	2.1
18	1,6-ctadiene,3,5-dimethyl-trans	$C_{10}H_{18}O$	164.2	–	–	2.7
19	Pseudowiddrene	$C_{15}H_{24}$	222.4	–	–	2.4
20	Cycloisolongifolene,8,9-dehydro-	$C_{15}H_{22}$	202.3	–	–	6.3
21	Caryophyllene oxide	$C_{15}H_{24}O$	220.4	–	–	4.2

Key
M^+ = Molecular Weight

The essential oil constituents namely, dihydrotagetone and limonene were present in all the oils of *T. minuta* obtained from the three zones. Of particular note was the high amount of dihydrotagetone in the essential oil of *T. minuta* obtained from Nyanza-Bondo zone. Differences in dihydrotagetone content in the essential oils of *T. minuta* have previously been attributed to differences in nitrogen content in the soils (Mohamed *et al*., 2004). Interesingly, *T. minuta* obtained from nitrogen deficient soils have higher percentage of dihydrotagetone content than the same cultivar grown in nitrogen sufficient soils (Singh *et al*., 2008). The composition of Nyanza-Bondo oil was significantly different from those of the other two with 2-pentanone (**21**), 2-hexanone (**22**), modheph-2-ene (**23**), pseudowiddrene (**24**), 8,9-dehydrocycloisolongifolene (**25**) and caryophyllene oxide (**26**) as its major constituents.



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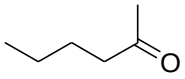

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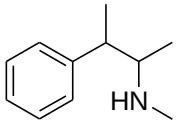

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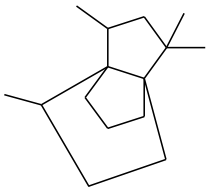

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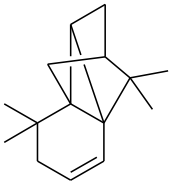

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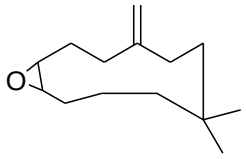

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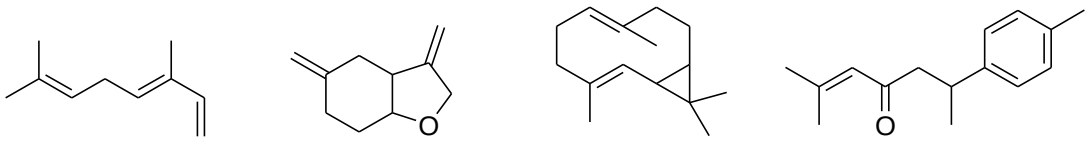
These differences in composition of the essential oils of *T. minuta* have been corroborated by GC and GC-MS analyses carried out previously on the essential oil of *T. minuta* collected from Bungoma in western Kenya which showed that the major

constituent was *cis*-ocimene (**27**) (43.78 %), followed by dihydrotagetone (16.71 %), piperitenone (10.15 %), *trans*-tagetone (8.67 %), 3,9-epoxy-*p*-metha-1,8(10)diene (**28**) (6.47 %), β-ocimene (3.25 %), *cis*-tagetone (1.95 %), caryophyllene oxide (0.84 %), bicyclogermacrene (**29**) (0.62 %) and AR-turmerone (**30**) (0.50 %) (Wanzala, 2009).

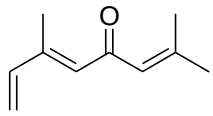


27 **28** **29** **30**

GC-MS analyses of the essential oils of *T. minuta* growing in UK, Egypt and South Africa has shown variations in chemical compositions. In UK, *T. minuta* was sampled from the field and a greenhouse of which the predominant components where dihydrotagetone, *cis*-tagetone and *trans*-tagetone, which together constituted 74.4 and 73.8 % of the total essential oils, respectively (Mohamed *et al*., 2004). Samples from UK had higher percentage content of dihydrotagetone (UK-greenhouse, 54.1 % and UK-field, 34.3 %) than those in Egypt and South Africa samples (3.0 and 16.4 %, respectively (Mohamed *et al*., 2004).

However, in these (from plants growing Egypt and South Africa) oils, *cis*-ocimene was the predominant product (50.9 and 32.0 %, respectively), but this was present at only low proportion in the UK samples. Similarly, *cis*-ocimenone (**31**) and *trans*-ocimenone were prominent components of the oils from Egypt and South Africa (16.5 and 9.8 %, respectively) but were present at very low concentrations (0.6 and 0.1 %) in the UK samples (Mohamed *et al*., 2004). In India, the freshly distilled *T. minuta* oil contained β-

ocimene (54.97 %) and dihdrotagetone (32.58 %) as the major constituents (Singh *et al*., 1992) and in Saudi Arabia, GC and GC/MS analysis showed that the major components in the essential oil of *T. minuta* were *cis*-tagetone (11.52 %), 2,7-dimethyl-5-octyn-4-one (**32**) (11.52 %), dicyclohexylpropanedinitrile (**33)** (10.45 %) and 2-pinen-4-one (**34**) (8.03 %) (El Deeb *et al*., 2004).



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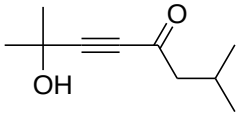

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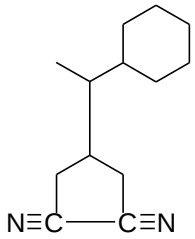

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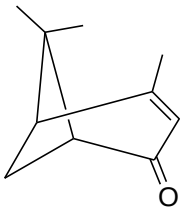

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The GC-MS analyses of the essential oils of *T. minuta* collected from three agro-ecological zones in Kenya gave different chromatograms (Figures 10a, 10b and 10c) where each peak represents the signal created when a compound in the injected essential oil elutes from the GC column to the detector. The peaks labeled correspond to the major constituents in each chromatogram as listed in Table 1.

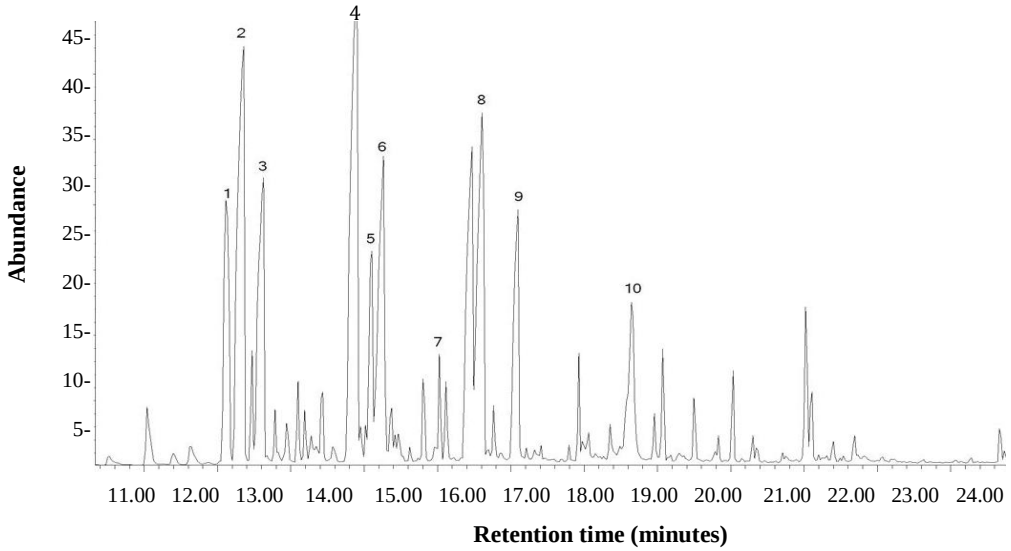


Figure 10a: GC-MS chromatogram of the essential oil *T. minuta* obtained from Nairobi-Kasarani zone

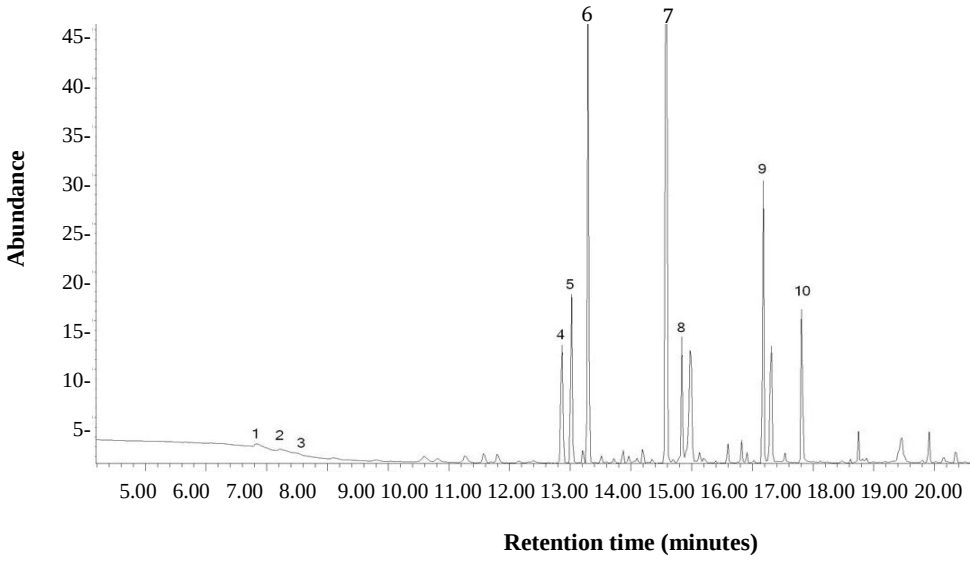


Figure 10b: GC-MS chromatogram of the essential oil of *T. minuta* obtained from western-Bungoma zone

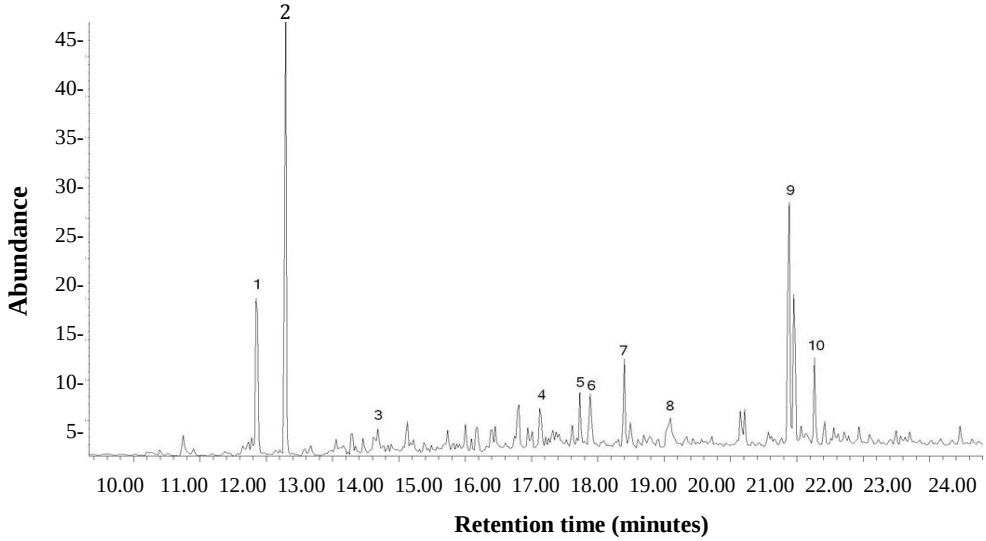


Figure 10c: GC-MC chromatogram of the essential oil of *T. minuta* obtained from Nyanza-Bondo zone

4.3 Evaluation of repellency of the essential oils of *T. minuta* using a dual-choice assay

The results of the dose-dependent response of newly emerged adult *R. appendiculatus* to the essential oils of *T. minuta* obtained from three different agro-ecological zones in a dual-choice tick climbing assay apparatus are summarized in Tables 2, 3, 4, 5, 6, 7 and 8. For a given dose and time of observation, there were varying degrees of dose- and time-dependent responses, respectively.

After 45 minutes of observation, there was a significant difference between mean percentages of repellency caused by different doses of essential oil of *T. minuta* obtained from Nairobi-Kasarani zone ($p = 0.002$) (Table 2), but there was no significant difference between mean percentages of repellency caused by the essential oils of *T. minuta* obtained from western-Bungoma ($p = 0.160$) and Nyanza-Bondo ($p = 0.215$) zones (Tables 4 and 6, respectively). After the 60^{th} minute's observation, there was a significant difference between the mean percentages of repellency caused by different doses of the essential oils of *T. minuta* from Nairobi-Kasarani and western-Bungoma zones ($p < 0.05$). However, there was no significant difference between the mean percentages of repellency caused by different doses of the essential oil of *T. minuta* from Nyanza-Bondo zone ($p > 0.05$) except at the 120^{th} minute observation ($p = 0.011$). Also, there was no significant difference between the mean percentages of repellency caused by the lower doses and the highest dose (2 mg) of the three essential oils of *T. minuta* over time ($p > 0.05$).

The overall mean percentages of repellency caused by the lower dose (0.5 mg) and highest dose (2 mg) of the three essential oils of *T. minuta* were significantly different (p

< 0.05) as shown in Figure 11. There were no significant time-course differences in the repellent effects of the essential oils ($p > 0.05$).

Family multiple comparisons between the overall mean percentages of repellency of the essential oil of *T. minuta* obtained from three different agro-ecological zones using a dual-choice assay ($n = 180$) is shown in Table 8. The essential oil of *T. minuta* from Nairobi-Kasarani zone had the highest overall mean percentange of repellency (70.76±2.78) relative to those associated with the essential oils from western-Bungoma (60.57±2.74) and Nyanza-Bondo (53.26±3.81) zones. Also, the overall mean percentage of repellency of the essential oil of *T. minuta* obtained from Nairobi-Kasarani zone was significantly different from the means of the essential oils from western-Bungoma and Nyanza-Bondo zones ($p < 0.05$) but the overall mean percentages of repellency of the essential oil of *T. minuta* obtained from western-Bungoma and Nyanza-Bondo zones were not significantly different ($p > 0.05$) (Wanzala, 2009).

Table 2: The mean (±SE) percentage of repellency caused by *T. minuta* essential oil obtained from Nairobi-Kasarani zone over time at different doses using the dual-choice assay (n = 10)

Doses (mg)	Time (minutes)						
	45	60	75	90	105	120	p-values
0.5	39.00±10.48^{a1}	44.33±9.86^{a1}	44.33±9.86^{a1}	38.33±10.60^{a1}	40.00±10.33^{a1}	44.00±11.85^{a1}	0.986
1	70.00±15.28^{ab1}	78.33±9.29^{b1}	66.67±14.05^{ab1}	81.00±7.81^{b1}	73.48±12.56^{b1}	83.33±11.39^{b1}	0.913
2	100.00±0.00^{b1}	96.00±4.00^{b1}	96.00±4.00^{b1}	93.33±6.67^{b1}	90.00±6.67^{b1}	96.00±4.00^{b1}	0.785
p-values	0.002	0.003	0.005	0.000	0.009	0.002	

Within a column, means (±SE) with the same letter(s), and across a given row, means with the same number(s) after the letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test), respectively.

Table 3: Overall mean (±SE) percentage of repellency of the essential oil of *T. minuta* from Nairobi-Kasarani zone obtained using a dual-choice assay

Doses (mg)	Mean (±SE) percentage of repellency
0.5	41.67 ±4.12^{a}
1	75.39±5.00^{b}
2	95.22±1.91^{c}
p-value	< 0.05

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)

Table 4: The mean (±SE) percentage of repellency caused by *T. minuta* essential oil obtained from western-Bungoma zone over time at different doses using the dual-choice assay (n = 10)

Doses (mg)	Time (minutes)						
	45	60	75	90	105	120	p-values
0.5	31.00±11.77^{a1}	32.00±12.89^{a1}	37.33±9.87^{a1}	29.33±7.08^{a1}	31.33±7.81^{a1}	36.33±11.93^{a1}	0.935
1	64.00±11.37^{b1}	73.00±9.55^{b1}	58.67±10.54^{ab1}	54.00±9.76^{ab1}	69.67±10.64^{b1}	81.33±9.98^{b1}	0.469
2	77.33±9.43^{b1}	80.00±10.75^{b1}	83.00±9.20^{b1}	82.00±7.42^{b1}	80.33±6.35^{b1}	89.33±7.38^{b1}	0.879
p-values	0.160	0.011	0.011	0.000	0.002	0.002	

Within a column, means (±SE) with the same letter(s), and across a given row, means with the same number(s) after the letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test), respectively.

Table 5: Overall mean (±SE) percentage of repellency of the essential oil of *T. minuta* from western-Bungoma zone obtained using a dual-choice assay

Doses (mg)	Mean (±SE) percentage of repellency
0.5	32.89±4.10^{a}
1	66.78±4.20^{b}
2	82.06±8.53^{c}
p-value	< 0.05

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test).

Table 6: The mean (±SE) percentage of repellency caused by *T. minuta* essential oil obtained from Nyanza-Bondo zone over time at different doses using the dual-choice assay (n = 10)

Doses (mg)	Time (minutes)						
	45	60	75	90	105	120	p-values
0.5	26.33±10.16^{a1}	32.33±14.67^{a1}	26.33±13.77^{a1}	24.67±11.32^{a1}	24.67±20.15^{a1}	25.00±14.83^{a1}	0.995
1	53.33±17.53^{b1}	58.67±17.35^{b1}	62.00±15.62^{b1}	50.00±22.91^{ab1}	52.67±17.95^{b1}	76.67±15.75^{b1}	0.909
2	65.00±15.80^{b1}	68.33±13.71^{b1}	78.33±15.72^{b1}	75.00±14.32^{b1}	73.33±9.03^{b1}	82.00±9.52^{b1}	0.559
p-values	0.215	0.246	0.061	0.129	0.123	0.011	

Within a column, means (±SE) with the same letter(s), and across a given row, means with the same number(s) after the letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test), respectively.

Table 7: Overall mean (±SE) percentage of repellency of the essential oil of *T. minuta* from Nyanza-Bondo zone obtained using a dual-choice assay

Doses (mg)	Mean (±SE) percentage of repellency
0.5	26.56±5.81^{a}
1	59.56±7.12^{b}
2	73.67±5.25^{b}
p-value	< 0.05

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test).

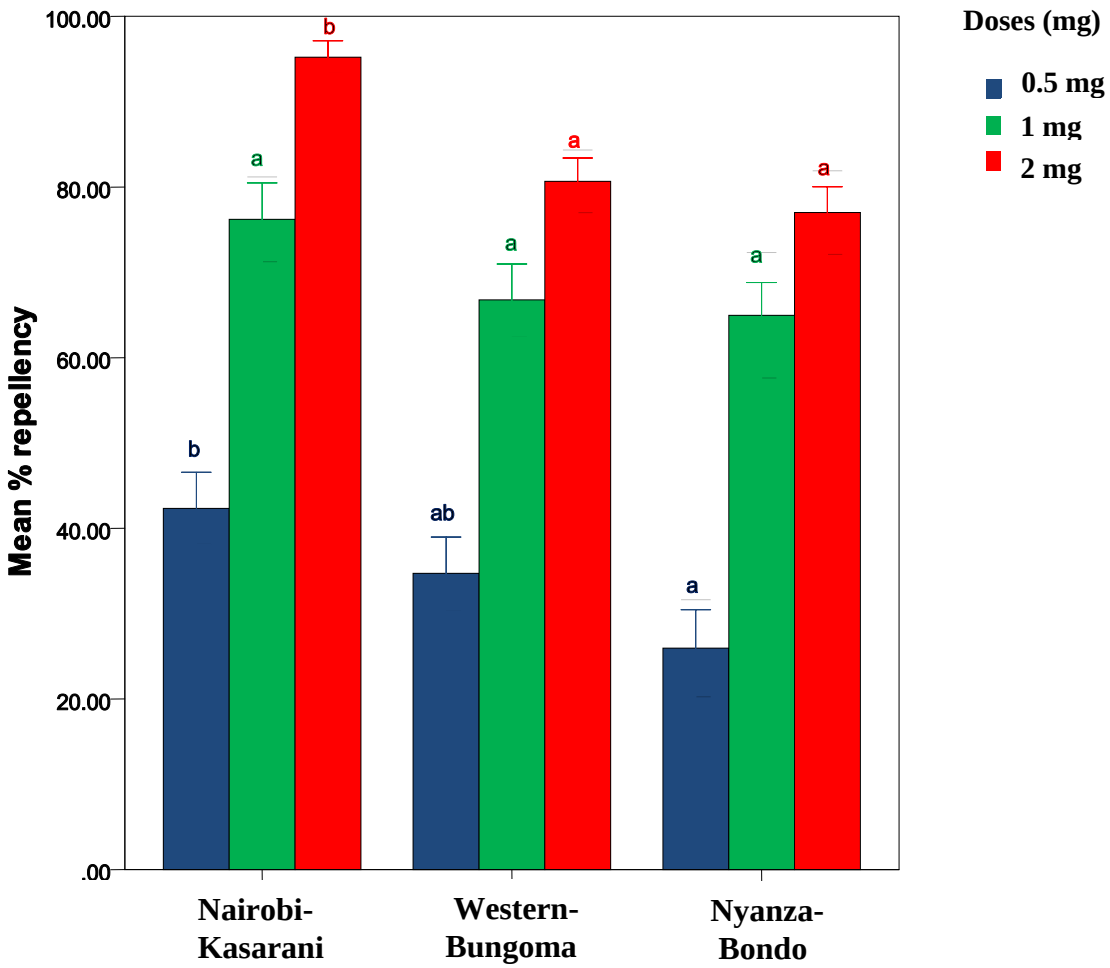


Figure 11: Comparison of the overall mean (±SE) percentage repellency caused by the essential oils of *T. minuta* obtained from three different agro-ecological zones (bars of the same color capped with the same small letters are not significantly different at α = 0.05 (Student-Newman-Keuls test).

Table 8: Family pairwise multiple comparisons between the overall mean (±SE) percentage repellency of the essential oils of *T. minuta* obtained from three different agro-ecological zones using dual-choice assays (n = 180)

Essential oils	Mean (±SE) percentages of repellency
Nyanza-Bondo	53.26±3.81[a]
Western-Bungoma	60.57±2.74[a]
Nairobi-Kasarani	70.76±2.78[c]

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test).

From these results, the essential oils of *T. minuta* obtained from the three agro-ecological zones showed a significant repellency against *R. appendiculatus* with that from Nairobi-Kasarani zone showing the highest repellency. Throughout the time of observation of the bioassays, the Nairobi-Kasarani essential oil maintained higher repellency at different doses than the other essential oils (Table 2). The results of the present study are also comparable to the earlier reports on the anti-tick repellent activities of the essential oil of *T. minuta* from Bungoma in western Kenya against *R. appendiculatus* (Wanzala, 2009).

The essential oil of *T. minuta* from western-Bungoma zone showed higher repellency than the oil from Nyanza-bond zone at all doses (Tables 5 and 7). The repellency of the essential oils increased with increase in the concentration of the doses. This assay did not show a clear trend of time-dependent responses of the adult *R. appendiculatus* to the essential oil of *T. minuta* obtained from three different agro-ecological zones. However, the assay shows a clear trend of dose-dependent tick climbing responses (Tables 3, 5 and

7). The key variables of this study, time (minutes), dose (mg), repellency and agro-ecological zone where *T. minuta* was obtained from were evaluated and found that repellency depends mainly on the dose (mg) and the agro-ecological zone because throughout the time of observation, duration of the assays did not show any significant difference in the responses of the ticks.

The repellency design of this experiment takes a regression model of:

$Repellency = \beta_0 + \beta_1 dose_i + \beta_2 zone_i + \varepsilon_i$ (Wanzala, 2009).

whereby, β_0 is the intercept constant and

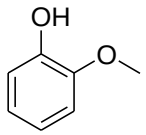
ε_i is the standard error term.

In this regression model, repellency is predicted by the dose and agro-ecological zone. Time as a variable was not statistically significant ($p > 0.05$) and, therefore, could not be considered as one of the predictors of the repellency in the regression model. The repellency link function of the regression model was therefore:

$Repellency = 0.451 dose_i - 0.167 zone_i + 37.0005$

4.4 Evaluation of repellency of guaiacol, β-ocimene and allo-ocimene individual constituents using a dual-choice assay

Guaiacol (**35)** is an oily naturally occurring aromatic organic compound. It is used as a dye in chemical reactions, as oxygen will turn guaiacol from colorless to brown. In a study with different structural analogues of guaiacol, 4-methyl guaiacol showed potent repellency to savannah tsetse fly (*Glossina spp*.) (Saini and Hassanali, 2007).



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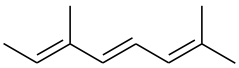
β-Ocimene is a monoterpene, which is a colorless liquid. It is employed as a botanical insecticide and used as a solvent for cleaning purposes (Odalo *et al*., 2005). It has been reported that β-ocimene together with dihydrotagetone isolated from the essential oil of *T. minuta* are potential sources of botanicals to control root-knot nematode *Meloidogyne incognita*. β-Ocimene has also been reported to have repellent activity against mosquito, *Anopheles gambiae* (Odalo *et al*., 2005).

Allo-ocimene (**36)** is a monoterpene, which is a clear colorless to slightly yellowish liquid. It is used in the creation and/or manufacturing of flavor and fragrance agents, and, to a small extent, in the perfume industry. It is also used as a diluting agent for varnishes and dyes and as a component for terpene polymers.



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The results of the dose-dependent response of these individual constituents against the newly emerged adult *R. appendiculatus* in a dual-choice tick climbing assay apparatus are shown in Tables 9, 10, 11, 12, 13, 14 and 15. For a given dose and time of observation, there were varying degrees of dose- and time-dependent responses, respectively.

In the first 45 minutes of observation, there was no significant difference between the mean percentages of repellency caused by different doses of allo-ocimene and β-ocimene ($p = 0.214$ and $p = 0.213$, respectively) (Tables 11 and 13) but guaiacol showed a significant difference between the mean percentages of repellency throughout the different doses (Tables 9). Thereafter, with the exception of 75^{th} minute of β-ocimene observation ($p = 0.071$), the mean percentages of repellency caused by different doses were significantly different ($p < 0.05$). Also, except at the 120^{th} minute of allo-ocimene observation ($p = 0.018$), the mean percentages of repellency caused by different doses were not significantly different ($p > 0.05$).

There was no significant difference between the mean percentages of repellency caused by lower doses and the highest dose (2 mg) of the individual constituents over time ($p > 0.05$). However, the overall mean percentages of repellency caused by different doses of the individual constituents were significantly different at different doses ($p < 0.05$) (Figure 12).

Family pairwise multiple comparisons between the overall mean percentages of repellency of guaiacol, β-ocimene and allo-ocimene individual constituents using a dual-choice assay ($n = 180$) is given in Table 15. Guaiacol showed the highest overall mean percentange of repellency (76.26±2.21), followed by β-ocimene (60.34±2.47) and allo-ocimene (40.67±3.36). The overall mean percentages of repellency of guaiacol, β-ocimene and allo-ocimene were significantly different ($p < 0.05$).

Table 9: The mean (±SE) percentage of repellency caused by guaiacol over time at different doses using the dual-choice assay (n = 10)

Doses (mg)	Time (minutes)						
	45	60	75	90	105	120	p-values
0.5	48.00±7.72^{a1}	54.33±6.57^{a1}	52.00±7.12^{a1}	53.00±7.16^{a1}	53.00±7.16^{a1}	56.00±11.04^{a1}	0.910
1	81.00±10.80^{b1}	72.00±12.27^{ab1}	71.33±12.57^{ab1}	79.00±7.06^{ab1}	82.00±9.52^{b1}	91.00±6.05^{b1}	0.707
2	100.00±0.00^{b1}	96.00±4.00^{b1}	96.00±4.00^{b1}	96.00±4.00^{b1}	96.00±4.00^{b1}	96.67±4.00^{b1}	0.700
p-values	0.000	0.006	0.005	0.000	0.001	0.002	

Within columns, means (±SE) with the same letter(s), and across a given row, means with the same number(s) after the letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test), respectively.

Table 10: Overall mean (±SE) percentage of repellency of guaiacol over time at different doses obtained using a dual-choice assay

Doses (mg)	Mean (±SE) percentage of repellency
0.5	52.72±3.11^{a}
1	79.39±4.01^{b}
2	96.67±1.44^{c}
p-value	< 0.05

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)

Table 11: The mean (±SE) percentage of repellency caused by allo-ocimene over time at different doses using a dual-choice assay (n = 10)

Doses (mg)	Time (minutes)						
	45	60	75	90	105	120	p-values
0.5	16.00±9.48^{a1}	17.00±10.45^{a1}	17.00±9.20^{a1}	22.00±11.28^{a1}	16.67±17.35^{a1}	20.00±10.97^{a1}	0.795
1	33.33±14.91^{a1}	40.00±13.17^{ab1}	50.00±14.68^{b1}	33.33±21.66^{a1}	46.67±16.44^{b1}	57.00±14.69^{b1}	0.853
2	51.00±15.75^{b1}	61.67±13.62^{b1}	52.00±17.44^{b1}	64.33±8.83^{b1}	65.33±7.91^{b1}	69.00±9.12^{b1}	0.354
p-values	0.213	0.057	0.166	0.137	0.075	0.018	

Within columns, means (±SE) with the same letter(s), and across a given row, means with the same number(s) after the letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test), respectively.

Table 12: Overall mean (±SE) percentage of repellency of allo-ocimene over time at different doses obtained using a dual-choice assay

Doses (mg)	Mean (±SE) percentage of repellency
0.5	18.11± 4.61^{a}
1	43.39±6.41^{b}
2	60.56±5.02^{c}
p-value	< 0.05

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)

Table 13: The mean (±SE) percentage of repellency caused by β-ocimene over time at different doses using a dual-choice assay (n = 10)

Doses (mg)	Time (minutes)						
	45	60	75	90	105	120	p-values
0.5	38.00 ±10.41^{a1}	32.00±12.89^{a1}	43.13±7.78^{a1}	35.00±8.87^{a1}	56.33±8.87^{a1}	45.13±9.37^{a1}	0.795
1	56.00±9.68^{b1}	69.00±9.12^{b1}	54.67±9.51^{ab1}	50.00±8.39^{a1}	61.67±9.37^{ab1}	69.33±9.33^{ab1}	0.853
2	66.00±12.93^{b1}	76.00±10.67^{b1}	78.00±9.52^{b1}	75.33±7.06^{b1}	80.33±8.53^{b1}	75.78±8.39^{b1}	0.354
p-values	0.214	0.019	0.071	0.002	0.040	0.003	

Within columns, means (±SE) with the same letter(s), and across a given row, means with the same number(s) after the letter(s) are not significantly different at α = 0.05 (Student-Newman-Keuls test), respectively.

Table 14: Overall mean (±SE) percentage of repellency of β-ocimene over time at different doses obtained using a dual-choice assay

Doses (mg)	Mean (±SE) percentage of repellency
0.5	45.13±4.37^{a}
1	60.11±3.75^{b}
2	75.78±3.84^{c}
p-value	< 0.05

Within a given column, means (±SE) with the same letter(s) are not significantly different at α = 0.05 (Student-Newman-Keuls test)

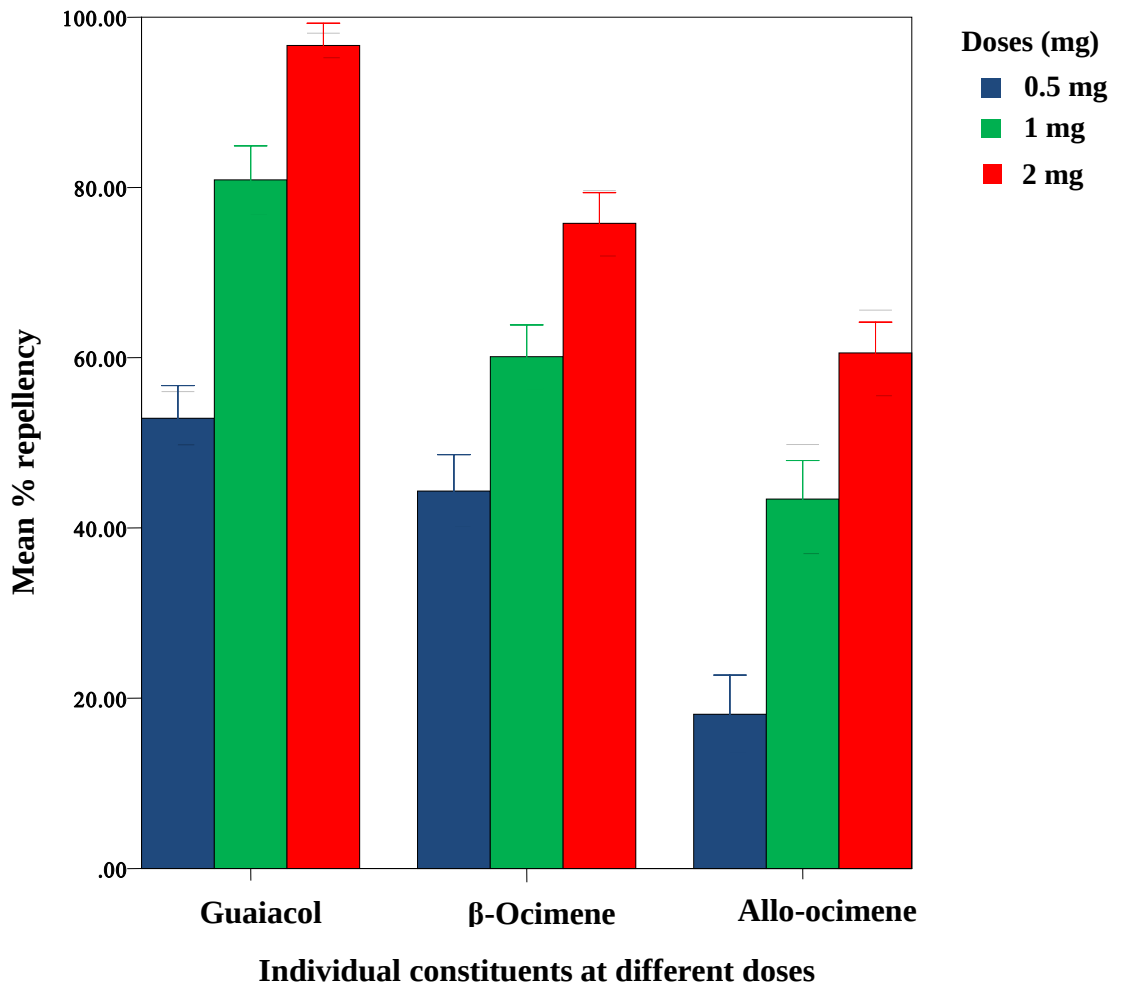

Figure 12: Comparison of the overall mean (±SE) percentage repellency caused by guaiacol, β-ocimene and allo-ocimene as individual constituents (bars of the same color are significantly different at α = 0.05 (Student-Newman-Keuls test).

Table 15: Family pairwise multiple comparisons between the overall mean (±SE) percentages of repellency of guaiacol, β-ocimene and allo-ocimene individual constituents using a dual-choice assay (n = 180)

Constituents	Mean (±SE) percentages of repellency
Allo-ocimene	40.67±3.36[a]
β-Ocimene	60.34±2.47[b]
Guaiacol	76.26±2.21[c]

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test).

From these bioassay results, the individual constituents showed significant repellencies against *R. appendiculatus* with guaiacol being the most repellent followed by β-ocimene and then allo-ocimene (Table 15). Throughout the period of the bioassays, guaiacol maintained higher repellency at different doses than the other constituents against *R. appendiculatus* (Table 9). The repellent results of guaiacol were comparable with those of the parent essential oil obtained from Nairobi-Kasarani zone at 1 and 2 mg doses (Tables 3 and 10). The repellency of the constituents increased with increase in the concentration of the doses. However, this assay did not show a clear trend of time-dependent responses of the adult *R. appendiculatus* to the constituents but the assay showed a clear trend of dose-dependent tick climbing responses (Tables 10, 12 and 14). The key variables of this study, time (minutes), dose (mg), repellency and the constituents were evaluated and found that repellency depends mainly on the dose (mg) and the particular constituent used in the bioassay because throughout the time of observation, time did not show any significant difference in repellency. Thus, the ticks appear to respond to these repellents as soon as they perceive them.

The repellency design of this experiment takes a regression model of:

$$\text{Repellency} = \beta_0 + \beta_1\, \text{dose}_i + \beta_2\, \text{constituent}_i + \varepsilon_i,$$

whereby, β_0 is the intercept constant and

ε_i is the standard error term.

In this regression model, repellency is predicted by the dose and the particular constituent used during bioassay. Time as a variable was not statistically significant ($p > 0.05$) and therefore could not be considered as one of the predictors of the repellency in the regression model. The repellency link function of the regression model was therefore:

$$\text{Repellency} = 0.392\text{dose}_i - 0.375\text{constituent}_i + 54.710$$

4.5 Evaluation of repellency of the blends using a dual-choice assay

The results of the dose-dependent response of newly emerged adult *R. appendiculatus* to the blends in a dual-choice tick climbing assay apparatus are shown in Tables 16, 17, 18, 19, 20, 21, 22, 23 and 24. For a given dose and time of observation, there were varying degrees of dose- and time-dependent responses, respectively.

There was a significant difference between mean percentages of repellency caused by different doses of β-ocimene, guaiacol and alloocimene (OGA),and β-ocimene and guaiacol (OG) blends ($p < 0.05$) with the exception of the 45^{th} and 75^{th} minute of observation ($p = 0.119$ and $p = 0.051$, respectively) (Tables 16 and 18). The mean percentages of repellency caused by different doses of guaiacol and alloocimene (GA)

blend were not significantly different ($p > 0.05$) with the exception of 75^{th} and 105^{th} minute of observation ($p = 0.005$ and $p = 0.021$, respectively) (Table 22).

Also, with the exception of 90^{th} and 120^{th} minute of observation ($p = 0.017$ and $p = 0.049$, respectively), there was no significant difference between the mean percentages of repellency caused by different doses of β-ocimene and alloocimene (OA) blend ($p > 0.05$) (Table 20). There was no significant difference between the mean percentages of repellency caused by lower doses and the highest dose (2 mg) of the four blends over time ($p > 0.05$). The overall mean percentages of repellency caused by different doses of OGA, OG and GA blends were not significantly different ($p > 0.05$) but they were significantly different from the overall mean percentages of repellency of OA blend ($p < 0.05$) (Figure 13).

Family pairwise multiple comparisons between the overall mean percentages of repellency of OGA, OG, OA and GA blends using a dual-choice assay ($n = 180$) is presented in Table 24. GA blend showed the highest overall mean percentange of repellency (75.20±2.87) than OG (70.13±2.80), OGA (67.33±2.42) and OA (50.39±3.20) blends. There was no significant difference between the overall mean percentages of repellency of OGA, OG and GA blends ($p > 0.05$). However, the overall mean percentage of repellency of OA blend was significantly different compared to the means of OGA, OG and GA blends ($p < 0.05$).

Table 16: The mean (±SE) percentage of repellency caused by OGA blend over time at different doses using the dual-choice assay (n = 10)

Doses (mg)	Time (minutes)						
	45	60	75	90	105	120	p-values
0.5	44.00±12.13^{a1}	43.33±7.25^{a1}	54.33±6.57^{a1}	43.33±9.74^{a1}	44.00±5.17^{a1}	39.00±9.12^{a1}	0.877
1	71.00±13.20^{ab1}	65.33±12.39^{ab1}	69.33±11.37^{a1}	76.00±8.05^{b1}	78.67±9.15^{b1}	80.33±8.35^{b1}	0.818
2	86.00±7.18^{b1}	85.33±7.81^{b1}	77.00±7.75^{a1}	83.33±8.61^{b1}	86.67±8.89^{b1}	85.00±7.64^{b1}	0.962
p-values	0.040	0.015	0.199	0.023	0.001	0.001	

Within columns, means (±SE) with the same letter(s), and across a given row, means with the same number(s) after the letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test), respectively.

Table 17: Overall mean (±SE) percentage of repellency of OGA blend over time at different doses obtained using the dual-choice assay

Doses (mg)	Mean (±SE) percentage of repellency
0.5	44.67±3.43^{a}
1	73.44±4.20^{b}
2	83.89±3.15^{b}
p-value	< 0.05

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)

Table 18: The mean (±SE) percentage of repellency caused by OG blend over time at different doses using the dual-choice assay (n = 10)

Doses (mg)	Time (minutes)						
	45	60	75	90	105	120	p-values
0.5	44.00±12.13^{a1}	47.33±11.21^{a1}	49.00±11.10^{a1}	46.33±11.13^{a1}	46.00±11.47^{a1}	47.00±13.00^{a1}	1.000
1	70.00±15.28^{ab1}	74.00±11.94^{ab1}	66.07±14.05^{ab1}	71.00±10.90^{ab1}	69.33±13.60^{ab1}	79.33±11.44^{b1}	0.989
2	90.00±10.00^{a1}	91.00±6.05^{b1}	96.00±3.99^{b1}	92.00±5.33^{b1}	93.30±6.67^{b1}	90.00±6.67^{b1}	0.988
p-values	0.051	0.017	0.030	0.003	0.018	0.023	

Within columns, means (±SE) with the same letter(s), and across a given row, means with the same number(s) after the letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test), respectively.

Table 19: Overall mean (±SE) percentage of repellency of OG blend over time at different doses obtained using the dual-choice assay

Doses (mg)	Mean (±SE) percentage of repellency
0.5	46.61±4.57^{a}
1	71.72±5.09^{b}
2	92.06±2.63^{c}
p-value	< 0.001

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)

Table 20: The mean (±SE) percentage of repellency caused by OA blend over time at different doses using the dual-choice assay (n = 10)

Doses (mg)	Time(min)						
	45	60	75	90	105	120	p-values
0.5	31.67±13.21^{a1}	36.33±13.59^{a1}	28.33±13.16^{a1}	24.67±11.32^{a1}	23.33±20.52^{a1}	25.00±14.63^{a1}	0.987
1	51.33±19.37^{a1}	55.33±11.88^{a1}	53.33±11.58^{ab1}	56.33±10.99^{ab1}	61.67±13.94^{b1}	54.00±13.35^{ab1}	0.997
2	60.67±12.02^{a1}	59.33±7.67^{a1}	66.00±11.85^{a1}	74.00±11.94^{b1}	74.00±9.33^{b1}	72.00±10.41^{b1}	0.893
p-values	0.375	0.330	0.104	0.017	0.068	0.049	

Within columns, means (±SE) with the same letter(s), and across a given row, means with the same number(s) after the letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test), respectively.

Table 21: Overall mean (±SE) percentage of repellency of OA blend over time at different doses obtained using the dual-choice assay

Doses (mg)	Mean (±SE) percentage of repellency
0.5	28.22±5.66^{a}
1	55.33±5.40^{b}
2	67.61±4.24^{b}
p-value	< 0.001

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)

Table 22: The mean (±SE) percentage of repellency caused by GA blend over time at different doses using the dual-choice assay (n = 10)

Doses (mg)	Time (min)						
	45	60	75	90	105	120	p-values
0.5	54.67±13.47^{a1}	59.67±15.64^{a1}	42.33±10.84^{a1}	57.33±17.30^{a1}	56.00±15.72^{a1}	59.00±13.03^{a1}	0.999
1	72.00±20.70^{ab1}	74.00±9.33^{ab1}	74.33±8.79^{ab1}	83.33±8.61^{b1}	76.00±8.84^{ab1}	78.00±12.09^{b1}	0.990
2	90.00±10.00^{b1}	90.00±10.00^{b1}	95.00±5.00^{b1}	96.00±4.00^{b1}	100.00±0.00^{b1}	96.00±4.00^{b1}	0.878
p-values	0.284	0.220	0.005	0.067	0.021	0.062	

Within columns, means (±SE) with the same letter(s), and across a given row, means with the same number(s) after the letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test), respectively.

Table 23: Overall mean (±SE) percentage of repellency of GA blend over time at different doses obtained using the dual-choice assay

Doses (mg)	Mean (±SE) percentage of repellency
0.5	54.83±5.67^{a}
1	76.28±4.78^{b}
2	95.00±2.60^{c}
p-value	< 0.001

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)

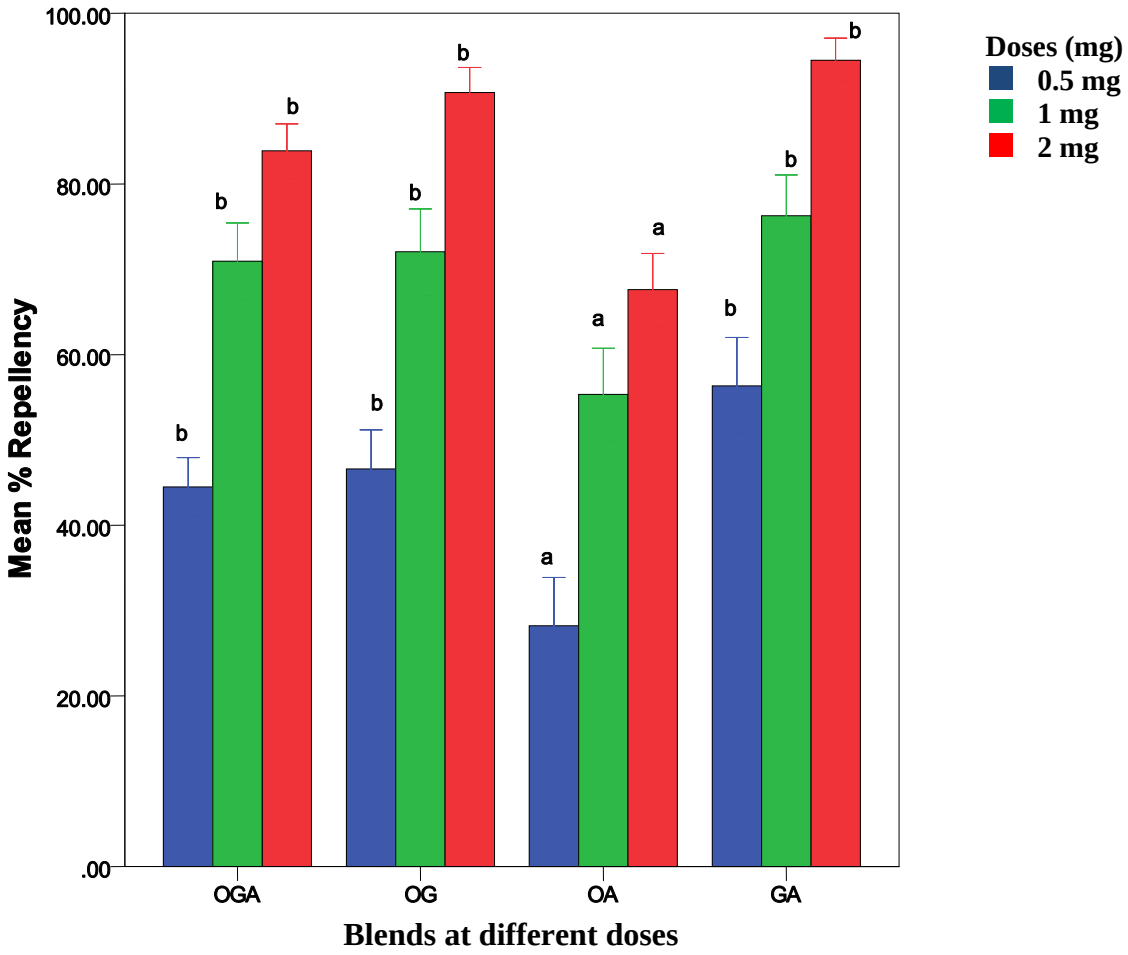

Figure 13: Comparison of the overall mean (±SE) percentage repellency caused by OGA, OG, OA and GA blends (bars of the same color capped with the same small letters are not significantly different at α = 0.05 (Student-Newman-Keuls test).

Table 24: Family pairwise multiple comparisons between the overall mean (±SE) percentage repellency of OA, OGA, OG and GA blends using a dual-choice assay (n = 180)

Constituent blends	Mean (±SE) percentages of Repellency
OA	50.39±3.20[a]
OGA	67.33±2.42[b]
OG	70.13±2.80[b]
GA	75.20±2.87[b]

Within a given column, means (±SE) with the same letter(s) are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test).

From these results of the four blends, all showed a significant repellency against *R. appendiculatus*. GA blend showed the highest repellency, followed by OGA, OG and OA blends. Throughout the time of observation of the bioassay, the GA blend maintained high repellency at different doses than the other three blends (Table 22). Also, both OGA and OG blends were more repellent than OA blend against *R. appendiculatus*. The repellency results of the GA blend at 1 and 2 mg doses were comparable to those of the parent oil obtained from Nairobi-Kasarani zone (Tables 3 and 23). Guaiacol as an individual constituent and GA blend showed comparable repellency at all doses (Tables 10 and 23, respectively). The repellency of the blends increased with increase in concentration of the dose. As in previous cases, this assay did not show a clear trend of time-dependent responses of the adult *R. appendiculatus* to the blends, but a clear trend of dose-dependent responses (Tables 17, 19, 21 and 23). The key variables of this study, dose (mg), time (minutes), repellency and type of blend were evaluated and found that

repellency depends mainly on the dose (mg) and the type of blend used in the bioassay because throughout the time of observation, time did not show any significant difference in repellency.

The repellency design of this experiment takes a regression model of:

$$\text{Repellency} = \beta_0 + \beta_1 \text{dose}_i + \beta_2 \text{blend}_i + \varepsilon_i,$$

whereby, β_0 is the intercept constant and

ε_i is the standard error term.

In this regression model, repellency is predicted by the dose and type of blend used in the bioassay. Time as a variable was not statistically significant ($p > 0.05$) and therefore could not be considered as one of the predictors of the repellency in the regression model. The repellency link function of the regression model was therefore:

$$\text{Repellency} = 0.275\text{dose}_i - 0.014\text{blend}_i + 38.329$$

A summary of multiple comparisons between the overall mean percentages of repellency of the essential oils of *T. minuta* obtained from three different agro-ecological zones, individual constituents and blends against *R. appendiculatus* using a dual-choice assay (n = 180) is given in Table 25. The overall mean percentage of repellency of allo-ocimene was significantly different from the means of other test materials ($p < 0.05$). Consequently, the overall mean percentages of repellency of OA blend, Nyanza-Bondo essential oil, β-ocimene and western-Bungoma essential oil were not significantly different (($p > 0.05$). Also, the overall mean percentages of repellency of β-ocimene,

western-Bungoma essential oil, OGA and OG blends and Nairobi-Kasarani essential oil were not significantly different ((p > 0.05). Similarily, the overall mean percentages of repellency of OGA and OG blends, Nairobi-Kasarani essential oil, GA blend and guaiacol were not significantly different ((p > 0.05).

Table 25: Multiple comparisons between the overall mean (±SE) percentages of repellency of all the tested materials (n = 180)

Test material	Means (±SE) of percentage repellency
Allo-ocimene	40.69 ± 3.36^{a}
OA blend	50.39 ± 3.20^{b}
Nyanza-Bondo essential oil	53.26 ± 3.81^{b}
β-Ocimene	60.34 ± 2.47^{bc}
Western-Bungoma essential oil	60.57 ± 2.74^{bc}
OGA blend	67.33 ± 2.42^{cd}
OG blend	70.13 ± 2.80^{cd}
Nairobi-Kasarani essential oil	70.76 ± 2.78^{cd}
GA blend	75.20 ± 2.87^{d}
Guaiacol	76.26 ± 2.21^{d}

Within a column, means (±SE) with a common superscript letter are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)

4.6 Determination of dose response of the essential oils of *T. minuta* using probit analysis

Dose-response relationships of the essential oils of *T. minuta* obtained from three different zones were determined using probit analyses and repellent doses (RD) at RD_{50} and RD_{75} values obtained from the regression model:

Probit [P(dose1)] = β_0 + x β_1 + Î,

whereby, β_0 = coefficient of the model representing y-intecept,

β_1 = coefficient of the model representing dose1,

dose1 = Log_{10} (dose),

Î = error term in the data set of the predictor variable (x) and

P = repellency probability.

The repellent doses (mg) of the essential oils obtained from the three zones at RD_{50} and RD_{75} levels are given in Table 26. The essential oil obtained from Nairobi-Kasarani showed low repellent dose at RD_{50} (0.00357 mg) and RD_{75} (0.07304 mg) levels than the essential oils obtained from western-Bungoma and Nyanza-Bondo zones. The dose-response link functions of the regression model predict what will be the repellent dose (mg) at a given repellence probability and for the essential oils they were:

Nairobi-Kasarani essential oil, Probit [P(dose1)] = 1.9785 + 0.6542 dose1,

Western-Bungoma essential oil, Probit [P(dose1)] = 0.7189 + 0.3946 dose1, and

Nyanza-Bondo essential oil, Probit [P(dose1)] = 0.6254 + 0.2462 dose1

Table 26: Probit analysis of dose-response relationship of the essential oils of *T. minuta* obtained from three different zones at RD_{50} and RD_{75}

Zones	Repellence probability	Repellent dose (mg)	Upper confidence limit at 95% level	Lower confidence limit at 95% level
Nairobi-Kasarani	0.50	0.00357	0.00392	0.00352
	0.75	0.07304	0.07449	0.07159
Western-Bugoma	0.50	0.27883	0.27908	0.27858
	0.75	0.83022	0.83165	0.82977
Nyanza-Bondo	0.50	0.60950	0.61095	0.60905
	0.75	0.90670	0.90715	0.90635

4.7 Determination of dose response of guaiacol, β-ocimene and allo-ocimene individual constituents using probit analysis

Dose-response relationships of guaiacol, β-ocimene and allo-ocimene individual constituents were determined using probit analyses and repellent doses at RD_{50} and RD_{75} values obtained from the regression model:

Probit [P(dose1)] = $\beta_0 + x\ \beta_1 + \hat{I}$

RD_{50} and RD_{75} values obtained from the repellency data of individual constituents are given in Table 27. RD_{50} and RD_{75} values of the constituents were not comparable to those of the parent oil obtained from Nairobi-Kasarani zone. Guaiacol had less RD_{50} (0.00112 mg) and RD_{75} (0.04876 mg) values while allo-ocimene showed high values impling that guaiacol as a constituent requires low repellent dose (mg) to repel *R. appendiculatus*. However, the RD_{50} and RD_{75} values of guaiacol were comparable to those of the GA

blend (Table 28). The dose-response link functions of the regression model for the individual constituents were therefore:

Guaiacol, Probit [P(dose1)] = 1.6959 + 0.4027 dose1,

β-Ocimene, Probit [P(dose1)] = 0.3406 + 0.1059 dose1, and

Allo-ocimene, Probit [P(dose1)] = 0.2198 + 0.0754 dose1

Table 27: Probit analysis of dose-response relationship of guaiacol, β-ocimene and allo-ocimene chemical constituents at RD_{50} and RD_{75}

Chemical constituents	Repellence probability	Repellent dose (mg)	Upper confidence limit at 95% level	Lower confidence limit at 95% level
Guaiacol	0.50	0.00112	0.00135	0.00089
	0.75	0.04876	0.04899	0.04853
β-Ocimene	0.50	0.40656	0.40680	0.40632
	0.75	0.87650	0.87670	0.87630
Allo-ocimene	0.50	1.00056	1.00079	1.00033
	0.75	2.45366	2.45389	2.45343

4.8 Determination of dose response of GA, OGA, OG and OA blends using probit analysis

Dose-response relationships of GA, OGA, OG and OA blends were determined using probit analyses and repellent doses at RD_{50} and RD_{75} values obtained from the regression model:

Probit [P(dose1)] = $\beta_0 + x\ \beta_1 + \hat{I}$

RD_{50} and RD_{75} values obtained from the repellency data of GA, OGA, OG and OA blends are given in Table 28. RD_{50} and RD_{75} values of the parent oil from Nairobi-Kasarani zone were comparable with those of OGA and OG blends but the values of GA and OA blends were not comparable to the ones of the parent oil. Thus from these values, GA blend requires a low repellent dose to repel *R. appendiculatus*. The dose-response link functions of the regression model for the blends were therefore:

GA blend, Probit [P(dose1)] = 1.1172 + 0.4224 dose1,

OGA blend, Probit [P(dose1)] = 1.3510 +0.1810 dose1,

OG blend, Probit [P(dose1)] = 1.8429 + 0.6132 dose1, and

OA blend, Probit [P(dose1)] = 1.0887 + 0.9707 dose1

Table 28: Probit analysis of dose-response relationship of GA, OGA, OG and OA blends at RD_{50} and RD_{75}

Constituent blends	Repellence probability	Repellent dose (mg)	Upper confidence limit at 95% level	Lower confidence limit at 95% level
GA	0.50	0.00134	0.00259	0.00209
	0.75	0.05659	0.05784	0.05634
OGA	0.50	0.00366	0.00389	0.00343
	0.75	0.07456	0.07479	0.07433
OG	0.50	0.00465	0.00486	0.00442
	0.75	0.08222	0.08245	0.08199
OA	0.50	0.97658	0.97679	0.97637
	0.75	1.54328	1.54349	1.54307

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

The GC-MS analyses of the essential oils of *T. minuta* collected from the three agro-ecological zones had markedly different compositions characterized by large variation in the proportion of guaiacol (Nairobi-Kasarani ~13.1 %, western-Bungoma 0 % and Nyanza-Bondo 0 %), and smaller variations in dihydrotagetone (Nairobi-Kasarani ~8.2 %, western-Bungoma ~8.7 % and Nyanza-Bondo ~9.5 %) and limonene (Nairobi-Kasarani ~4.3 % , western-Bungoma ~3.3 % and Nyanza-Bondo ~4.8 %) (Table 1). This indicates the existence of different chemotypes within Kenya. Chemotypic variations have been attributed to a number of factors: climatic and edaphic conditions under which the plants grow (temperature profile, humidity, rainfall and precipitation), growing season, and soil type and nutrient status.

The high activity of the essential oil of *T. minuta* obtained from Nairobi-Kasarani zone against *R. appendiculatus* appears to be associated with the presence of relatively high amounts of guaiacol, which showed high repellency compared with β-ocimene and allo-ocimene (Table 15). RD_{50} and RD_{75} of natural blends of the oil collected from different areas, individual constituents and synthetic blends assayed, show that guaiacol and guaiacol-alloocimene (GA) blend had the highest repellency (Table 26, 27 and 28).

The present study provides a useful basis for quality control of *T. minuta* essential oil that is exploited for tick control. Although the repellency of the oils obtained from the three agro-ecological zones differed, the variation was not large and all the three samples may have potential in tick control.

5.2 Recommendations

The following are recommended from this research;

i. A study to compare the effects of on-host (cattle) performance of controlled-release formulations at different doses of the three essential oils of *T. minuta* and selected blends (guaiacol-alloocimene) on infestations of *R. appendiculatus*.

ii. Screening of individual other constituents of the three essential oils of *T. minuta* and their blends against *R. appendiculatus* to find out if their contribution is significant.

iii. Detailed study of the relationship between ecological factors and chemotypic differences of *T. minuta* growing in different areas.

iv. Off-host and on-host comparison of the essential oil of *T. minuta* from specific areas with essential oils from other plants which have been found to have anti-tick properties against *R. appendiculatus* and the effects of combining these to explore any possible synergistic effects.

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APPENDICES

Appendix 1a: GC-MS constituent compounds identified in the essential oil of *T. minuta* obtained from Nairobi-Kasarani zone

No.	Compound	Molecular formula	M+ (g/mol)	RT (min)	Relative (%)
1.	2-Nonanone,9-[(tetrahydro-2H-pyran-2-yl)oxy]-	$C_{14}H_{26}O_3$	242.4	5.4	0.02
2.	Toluene	C_7H_8	92.1	6.0	0.02
3.	Furan, 2,3-dihydro-4-methyl	C_5H_8O	84.1	6.7	0.01
4.	2-Pentene, 3,4-dimethyl-, (E)-	C_7H_{14}	98.2	7.1	0.02
5.	3-Hexanol	$C_{10}H_{14}O$	102.2	7.4	0.03
6.	2-Butanone,3-methyl	$C_5H_{10}O$	82.0	7.7	0.02
7.	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222.5	7.8	0.01
8.	2-Butenoic acid, 3-methyl-2-ethylhexyl ester	$C_{13}H_{24}O_2$	212.3	8.2	0.01
9.	Ethyl-2-methyl butanoate	$C_7H_{14}O_2$	130.2	8.4	0.23
10.	2-Methylbutylacetate	$C_7H_{14}O_2$	130.2	9.0	0.07
11.	1H-Imidazole, 2-ethyl-4-methyl-	$C_6H_{10}N_2$	110.2	10.1	0.32
12.	Camphene	$C_{10}H_{16}$	136.2	10.6	0.18
13.	Sabinene	$C_{10}H_{16}$	136.2	11.1	0.74
14.	Myrcene	$C_{10}H_{16}$	136.2	11.4	0.17
15.	α-Phellandrene	$C_{10}H_{16}$	136.2	11.7	0.39

Appendix 1a; continued'

No.	Compound	Molecular formula	M^+ (g/mol)	RT (min)	Relative (%)
16.	β-Ocimene	$C_{10}H_{16}$	136.2	12.1	13.17
17.	Limonene	$C_{10}H_{16}$	136.2	12.5	4.31
18.	Dihydrotagetone	$C_{10}H_{18}O$	154.0	12.6	8.20
19.	Allo-ocimene	$C_{10}H_{16}$	136.2	13.9	10.15
20.	*cis*-Tagetone	$C_{10}H_{16}O$	152.2	14.1	2.89
21.	*trans*-Tagetone	$C_{10}H_{16}O$	152.2	14.3	9.39
22.	Methyl-2-methyl butyrate	$C_7H_{15}O$	115.0	14.7	0.07
23.	Verbenone	$C_{10}H_{14}O$	150.2	15.1	2.50
24.	β-Ocimenone	$C_{10}H_{14}O$	150.2	15.5	6.25
25.	5-Ethyl-4-methyl-2H-pyran-2-one	$C_8H_{10}O_2$	138.0	15.3	0.003
26.	Guaiacol	$C_7H_8O_2$	124.1	15.6	13.13
27.	Car-3-en-2-one	$C_{10}H_{14}O$	150.2	16.1	4.11
28.	Elsholtzia ketone	$C_{10}H_{14}O_2$	166.2	16.4	0.58
29.	β-Alaskene	$C_{15}H_{24}$	204.4	17.0	1.78
30.	Furan, 2,3,5, trimethyl	$C_7H_{10}O$	110.0	17.2	0.003
31.	Eucarvone	$C_{10}H_{14}O$	150.2	17.7	3.62
32.	β-Elemene	$C_{15}H_{24}$	204.4	18.1	1.52
33.	7-Propylidene-bicyclo [4.1.0] Heptane	$C_{10}H_{16}$	136.2	18.3	0.32
34.	α-Humulene	$C_{15}H_{24}$	204.4	18.5	0.63

Appendix 1a; continued'

No.	Compound	Molecular formula	M^+ (g/mol)	RT (min)	Relative (%)
35.	α-Pinene	$C_{10}H_{16}$	136.2	18.8	0.05
36.	Bicyclogermacrene	$C_{15}H_{24}$	204.4	19.0	1.80
37.	Acetic acid, tricycle [3.3.1.1(3,7)]decylidene- ,ethyl ester	$C_{14}H_{20}O_2$	220.3	19.8	0.39
38.	*cis*-.*alpha*.-Copaene-8-ol	$C_{15}H_{24}O$	220.4	20.7	0.62
39.	3-Heptyne,7-bromo- 2, 2- dimethyl	$C_9H_{15}Br$	203.2	21.1	0.49
40.	β-Pinene	$C_{10}H_{16}$	136.2	20.5	0.03
41.	N-Octanol	$C_8H_{16}O$	128.0	20.7	0.17
42.	3-Hexene-1-ol, acetate	$C_8H_4O_2$	142.0	20.9	0.02
43.	Phthalic acid, dodecyl ethyl ester	$C_{22}H_{35}O_4$	359.5	21.6	0.22
44.	*cis*-Ocimene	$C_{10}H_{16}$	136.2	22.7	0.07
45.	Hexadecanol	$C_{16}H_{34}O$	242.4	23.1	0.49
46.	Tricyclene	$C_{10}H_{16}$	136.2	23.5	0.02
47.	1H-Indole, 4-(3-methyl-2-butenyl)-	$C_{13}H_{15}N$	187.2	23.6	0.39
48.	α-Terpinolene	$C_{10}H_{16}$	136.2	23.6	0.01
49.	α-Citral	$C_{10}H_{16}O$	152.2	23.7	0.03
51.	Geraniol formate	$C_{11}H_{18}O_2$	182.0	24.6	0.04
52.	Linalool	$C_{10}H_{18}O$	154.3	24.7	0.07
53.	Phenyl-1,2-diamine, N,4,5-trimethyl-	$C_9H_{14}N_2$	150.2	25.0	2.62

Appendix 1a; continued'

No.	Compound	Molecular formula	M^+ (g/mol)	RT (min)	Relative (%)
54.	3-Methylbut-2-enoic acid,3,5-dimethyl phenylester	$C_{13}H_{16}O_2$	204.3	26.0	0.96
55.	2-sec-Butyl-4,6-dinitro phenyl 3-methyl crotonate (binapacryl)	$C_{15}H_{18}N_2O_6$	322.3	26.2	2.03
56.	3-Methyl-2-butenoic acid, 2,7-dimethyloct-7-en-5-yn-4-yl ester	$C_{15}H_{22}O_2$	234.3	27.2	0.41
57.	Benzo[b]naphtha[2,3-d] thiophene-6,8-dimethyl-	$C_{18}H_{15}S$	263.3	27.4	0.72
58.	α-Terpineol	$C_{10}H_{18}0$	154.3	27.9	0.02
59.	Naphthen-1-acetic acid, 8-methoxy-.*alpha*. methyl-	$C_{14}H_{14}O_3$	230.3	28.3	0.15
60.	Methyl dodecanoate	$C_{13}H_{26}O_2$	214.3	28.8	0.27
61.	Piperitenone	$C_{10}H_{16}O$	152.0	29.4	1.10
62.	Carvacrol	$C_{10}H_{14}O$	150.0	29.9	0.12
63.	Eicosene	$C_{20}H_{40}$	280.5	30.2	0.07
64.	Dihydroedulan II	$C_{13}H_{22}O$	194.0	31.7	0.03
65.	Hexacosane	$C_{26}H_{54}$	366.7	31.9	0.01
66.	Methylcyclohexyl carbonyl-.beta.-d-glucuronide, triacetate	$C_{20}H_{28}O_{11}$	444.4	32.9	0.06
67.	α-Guaiene	$C_{15}H_{24}$	204.0	33.6	0.05
68.	Eicosane	$C_{20}H_{42}$	282.6	34.5	0.09

Appendix 1a; continued'

No.	Compound	Molecular Formula	M^+ (g/mol)	RT (min)	Relative (%)
69.	Heptasiloxane, hexadecamethyl-	$C_{16}H_{48}O_6Si_7$	533.2	34.9	0.06
70.	3-Methyl-2-butenoicacid, 2,6-dimethylnon-1-en-3-yn-5-yl ester	$C_{16}H_{24}O_2$	248.4	35.3	0.34
71.	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667.4	37.6	0.20
72.	Valerenol	$C_{15}H_{22}$	202.0	39.1	0.02
73.	Spathulenol	$C_{15}H_{22}$	202.0	39.7	0.02
74.	Benzo[h]quinoline, 2,4-dimethyl-	$C_{15}H_{13}N$	207.3	40.9	0.18
75.	Megestrol Acetate	$C_{24}H_{32}O_4$	384.5	42.3	0.04
76.	6-Carbomethoxy-5,8-dimethoxy-1-tetralone	$C_{14}H_{16}O_5$	264.3	42.5	0.08
77.	Thiazolin-2-imine,3-(2-cyclohexenylethyl)-2(4-methoxyphenyl)-4- phenyl-	$C_{24}H_{26}N_2OS$	390.5	43.2	0.18
78.	3-Amino-7-nitro-1,2,4-benzotriazine-1-oxide	$C_7H_5N_5O_3$	207.1	44.4	0.05
79.	1H-Indole,2-methyl-3- phenyl-	$C_{15}H_{13}N$	207.3	44.8	0.13
80.	2-Amino-benzoic acid, N'-acridin-9-ylhydrazide	$C_{20}H_{15}N_3O$	313.4	45.6	0.07
81.	1-Benzopyrylium, 2- phenyl-	$C_{15}H_{11}O$	207.3	46.0	0.08
82.	β-Atlantone	$C_{10}H_{16}O$	152.0	47.9	0.02

Appendix 1b: GC-MS constituent compounds identified in the essential oil of *T. minuta* obtained from western-Bungoma zone

No.	Compound	Molecular formula	M^+ (g/mol)	RT (min)	Relative (%)
1.	2-Pentanone	$C_5H_{10}O$	86.1	3.3	25.28
2.	Ether,2-chloro-1-methylethylisopropyl	$C_6H_{12}Cl_2O$	171.2	6.8	3.83
3.	2-Hexanone	$C_6H_{12}O$	100.0	7.2	4.07
4.	Furan, 2,3,5-trimethyl-	$C_7H_{10}O$	110.2	9.8	0.31
5.	4-Nonanol, 4-methyl-	$C_{10}H_{22}O$	158.3	10.6	0.35
6.	Sabinene	$C_{10}H_{16}$	136.2	10.8	0.38
7.	Phellandrene	$C_{10}H_{16}$	136.2	11.4	0.20
8.	Limonene	$C_{10}H_{16}$	136.2	11.9	3.34
9.	β-Ocimene	$C_{10}H_{16}$	136.2	12.0	3.84
10.	Dihydrotagetone	$C_{10}H_{18}O$	154.0	12.3	8.73
11.	Allo-ocimene	$C_{10}H_{16}$	136.2	13.6	13.89
12.	*cis*-Tagetone	$C_{10}H_{16}O$	152.2	13.9	1.25
13.	*trans*-Tagetone	$C_{10}H_{16}O$	152.2	14.0	4.59
14.	1,2,3,6-Tetrahydro pyridin-2-carboxylic acid	$C_6H_9NO_2$	127.1	14.6	0.42
15.	2H-Pyrido[3,2-b]-1, 4-oxazin-3(4H)-one	$C_7H_6N_2O_2$	150.1	14.8	0.47
16.	β-Ocimenone	$C_{10}H_{14}O$	150.2	15.2	5.24
17.	Carvacrol	$C_{10}H_{14}O$	150.2	15.5	0.37
18.	2-Cyclohexen-1-ol, 3,5,5-trimethyl	$C_9H_{16}O$	140.2	15.8	3.56

Appendix 1b; continued'

No.	Compound	Molecular formula	M^+ (g/mol)	RT (min)	Relative (%)
19.	3-Hydroxyaceto-Phenone	$C_8H_8O_2$	136.2	16.8	0.72
20.	Eucarvone	$C_{10}H_{14}O$	150.2	17.5	1.65
21.	b-Elemene	$C_{15}H_{24}$	204.4	17.9	0.66
22.	Humulene	$C_{15}H_{24}$	204.4	18.4	0.38
23.	Germacrene D	$C_{15}H_{24}$	204.4	18.7	0.21
24.	Chamigrene	$C_{15}H_{24}$	204.4	18.9	0.32
25.	L-Proline-1-acetyl-	$C_7H_{10}NO_3$	156.2	19.2	0.65
26.	7-Methoxy-3,4,5,6-tetramethyl-2,1-benzisoxazole	$C_{12}H_{15}NO_2$	205.3	19.9	0.24
27.	Silphiperfol-4- en-5-one	$C_{15}H_{22}O$	218.3	20.4	0.06
28.	β-Pinene	$C_{10}H_{16}$	136.2	20.5	0.03
29.	Muurola-4,10(14)-dien-1-beta-ol	$C_{15}H_{24}O$	220.4	20.6	1.28
30.	Cyclopentasiloxane, decamethyl-	$C_{10}H_{30}O_5Si_5$	370.8	20.7	0.04
31.	Phenyl-1,3-diamine, N,4,5-trimethyl-	$C_9H_{14}N_2$	150.2	24.9	0.32
32.	1,1-Propanedicarbo-nitrile, 1,2-dicyclohexyl-	$C_{17}H_{26}N_2$	258.4	25.4	1.20
33.	4-Thiomethyl-5-amino Veratrole	$C_9H_{13}NO_2S$	199.3	25.9	0.56
34.	*cis*-Tagetone	$C_{10}H_{16}O$	152.3	26.1	1.85

Appendix 1b; continued'

No.	Compound	Molecular formula	M^+ (g/mol)	RT (min)	Relative (%)
35.	3-Methyl-2-butenoic acid, tridec-2-ynyl Ester	$C_{18}H_{30}O_2$	278.4	26.2	0.67
36.	Silane, trichloro-cyclohexyl-	$C_6H_{11}Cl_3Si$	217.6	26.7	1.80
37.	Dimethyl endo-borneol	$C_{10}H_{18}O$	154.3	27.2	0.02
38.	4-Formyl-3,5-di-t-butylbenzoic acid	$C_{16}H_{22}O_3$	262.4	27.4	0.08
39.	3-Cyclohexen-I-ol, 4-methyl	$C_{10}H_{18}O$	154.3	27.6	0.07
40.	Carvone	$C_{10}H_{14}O$	150.0	27.7	0.06
41.	N-Decanal	$C_{10}H_{16}O$	152.0	28.3	0.41
42.	3,9-Epoxy-p-metha-1,8(10) diene	$C_{10}H_{14}O$	150.0	29.1	1.67
43.	Azulene 4,5,6,7,8,7A	$C_{13}H_{22}O$	194.0	31.2	0.04
44.	Dihydroedulan I	$C_{13}H_{22}O$	194.0	31.7	0.02
45.	Bicycloelemene	$C_{15}H_{24}$	204.0	33.1	0.06
46.	Heptasiloxene, hexadecamethyl-	$C_{16}H_{46}O_6Si_7$	533.2	34.9	0.06
47.	3-Methyl-2-butenoic acid, 4, 6-dimethylnon-1- en-2-yn-5-yl ester	$C_{16}H_{24}O_2$	248.4	35.2	0.03
48.	Cyclononasiloxene, octadecamethyl-	$C_{18}H_{52}O_9Si_9$	667.4	37.4	0.20

Appendix 1b; continued'

No.	Compound	Molecular formula	M^+ (g/mol)	RT (min)	Relative (%)
49.	1,4-Benzenediol,2(1,1) dimethylethyl	$C_{15}H_{22}O_2$	234.0	38.2	0.03
50.	Aromadedrene	$C_{15}H_{22}$	202.0	40.2	0.02
51.	Isospathulenol	$C_{15}H_{22}$	202.0	41.0	0.05
52.	Delta- elemene	$C_{15}H_{22}$	202.0	41.5	0.03
53.	4-Thiazolin-2-imine,3-(2-cyclohexenylethyl)-2(4-methoxyphenyl)-4-phenyl-	$C_{24}H_{26}N_2OS$	390.5	42.5	0.01
54.	3-Amino-7-nitro-1,2,4-benzotriazine 1-oxide	$C_7H_5N_5O_3$	207.2	43.2	0.15
55.	Neophytadiene	$C_{20}H_{38}$	278.0	45.5	0.21
56.	4-Heptyn-3-ol	$C_7H_{12}O$	112.0	48.6	0.01
57.	α-Cedrol	$C_{15}H_{26}O$	222.0	49.0	0.03
58.	1,5,7-Octatriene-3-one, 2,6-dimethyl	$C_{15}H_{22}$	202.0	49.5	0.01
59.	E-ocimenone	$C_{10}H_{14}O$	150.2	50.2	0.22
60.	Cinamyl Tiglate	$C_{15}H_{20}O_2$	232.0	51.2	0.10

Appendix 1c: GC-MS constituent compounds identified in the essential oil of *T. minuta* obtained from Nyanza-Bondo zone

No.	Compound	Molecular formula	M^+ (g/mol)	RT (min)	Relative (%)
1.	(3Z)-Octen-2-ol	$C_8H_{16}O$	128.2	10.8	0.75
2.	Sabinene	$C_{10}H_{16}$	136.2	10.8	0.38
3.	Phellandrene	$C_{10}H_{16}$	136.2	11.4	0.20
4.	o*rtho*-Cymene	$C_{10}H_{14}$	134.2	11.8	0.82
5.	Limonene	$C_{10}H_{16}$	136.2	11.9	4.77
6.	β-Ocimene	$C_{10}H_{16}$	136.2	12.0	1.30
7.	Dihydrotagetone	$C_{10}H_{18}O$	154.0	12.3	9.46
8.	Verbenone	$C_{10}H_{14}O$	150.2	12.4	0.30
9.	α-Ocimenone	$C_{10}H_{14}O$	150.2	12.9	1.14
10.	Phenol, 2,3,5,6-tetramethyl-	$C_{10}H_{14}O$	150.2	13.1	0.96
11.	8-Oxabicyclo[3.2.1] oct-6-en-3-one	$C_7H_8O_2$	124.1	13.3	0.94
12.	2-Cyclohexen-1-ol, 3,5,5-trimethyl-	$C_9H_{16}O$	140.2	13.6	2.18
13.	Allo-ocimene	$C_{10}H_{16}$	136.2	13.8	1.01
14.	*cis*-Tagetone	$C_{10}H_{16}O$	152.2	14.1	0.19
15.	*trans*-Tagetone	$C_{10}H_{16}O$	152.2	14.3	1.05
16.	Thiophene, 2-ethyl-	C_6H_8S	112.2	14.3	1.94
17.	Elsholtzia ketone	$C_{10}H_{14}O_2$	166.2	14.7	1.28
18.	*trans*-Carveol	$C_{10}H_{16}O$	152.2	15.0	0.32

Appendix 1c; continued'

No.	Compound	Molecular formula	M^+ (g/mol)	RT (min)	Relative (%)
19.	Guanidine, N-(4-fluorophenyl)-	$C_{16}H_{13}FN_3$	153.2	15.2	0.97
20.	Carvone	$C_{10}H_{18}O$	150.0	15.4	1.76
21.	8-Oxabicyclo[5.1.0]oct-2-en-4-one,3,6,6-trimethyl-	$C_{10}H_{14}O_2$	146.3	15.8	2.12
22.	3-Chloropropionic acid, tridec-2-ynyl ester	$C_{16}H_{27}ClO_2$	286.9	16.1	1.96
23.	1-(1-Hydroxy-1-methyl (-ethyl) -cyclobutane carboxylic acid	$C_8H_{17}O_3$	161.2	16.3	2.34
24.	Silphiperfol-5-ene	$C_{15}H_{24}$	204.4	16.6	1.06
25.	Naphth[2,3-b]oxirene, decahydro-	$C_{10}H_{16}O$	152.2	16.8	1.70
26.	Morpholine, 4 [3-(4-butoxyphenoxy) propyl]-	$C_{17}H_{27}NO_3$	273.2	16.9	2.31
27.	α-Alaskene	$C_{15}H_{24}$	204.4	17.0	0.68
28.	Modheph-2-ene	$C_{15}H_{24}$	204.4	17.4	2.14
29.	3-Pyridinecarbonitrile, 1,2-dihydro-4-methoxy-1- methyl-2-oxo-	$C_8H_8N_2O_2$	164.2	17.5	1.02
30.	Eucarvone	$C_{10}H_{14}O$	150.2	17.9	0.22
31.	α-Elemene	$C_{15}H_{24}$	204.4	18.1	0.02
32.	1,6-Octadiene, 3,5-dimethyl-trans	$C_{10}H_{18}O$	154.2	18.1	2.66

Appendix 1c; continued'

No.	Compound	Molecular formula	M^+ (g/mol)	RT (min)	Relative (%)
33.	α-Humulene	$C_{15}H_{24}$	204.4	18.3	0.03
34	Hexadecanol	$C_{16}H_{34}O$	242.4	18.9	0.02
35	Pseudowiddrene	$C_{15}H_{24}$	222.4	19.2	2.42
36	8,9-dehydro-cycloisolongifolene,	$C_{15}H_{22}$	202.3	19.9	6.31
37	Caryophyllene oxide	$C_{15}H_{24}O$	220.4	20.0	4.21
38	Humulene epoxide II	$C_{15}H_{24}O$	220.4	20.3	2.08
39	Silphiperfol-6-en-5-one	$C_{15}H_{22}O$	218.3	20.4	0.85
40	*alpha*.-*cis*-.Copaene-8-ol	$C_{15}H_{24}O$	220.4	20.5	0.03
41	Muurola-4,10(14)-dien-1-beta-ol	$C_{15}H_{24}O$	220.4	20.6	1.28
42	3-Heptyne,7-bromo-2,2- dimethyl-	$C_9H_{15}Br$	203.2	21.1	0.49
43	Phthalic acid, dodecyl ethyl ester	$C_{22}H_{35}O_4$	359.5	21.3	0.02
44	Phenyl-1,2-diamine,N,4,5–trimethyl-	$C_9H_{14}N_2$	150.2	25.0	2.62
45	1,1-Propanedicarbo-nitrile,1,2-dicyclohexyl-	$C_{17}H_{26}N_2$	258.4	25.4	1.20
46	1H-Indole,4-(3-methyl-2-butenyl)-	$C_{13}H_{15}N$	187.2	25.5	0.06
47	Silane, trichloro-cyclohexyl-	$C_6H_{11}Cl_3Si$	217.6	26.2	1.80